# The effect of aged gut microbiota transfer in Alzheimer's disease

Dissertation

zur Erlangung des Grades

„Doktor der Naturwissenschaften“

im Promotionsfach Chemie

am Fachbereich Chemie, Pharmazie, Geographie und Geowissenschaften

der Johannes Gutenberg-Universität Mainz

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Geboren am 29/08/1992 in Rom (Italien)

Mainz 2023

1. Gutachter:in:
2. Gutachter:in:

Tag der mündlichen Prüfung: 27/10/2023

This work was carried out between June 2018 and December 2021 under the supervision of [redacted] in the Biochemical Laboratory of the Department of Psychiatry and Psychotherapy of the University Medical Center of the Johannes Gutenberg-University (Mainz, Germany).

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I hereby declare, on oath, that I have written this dissertation independently and have not used any other aids/sources than those specified in this thesis. Furthermore, I declare that no other examinations procedure has been applied before or that this thesis has been submitted elsewhere as an examination thesis in another faculty.

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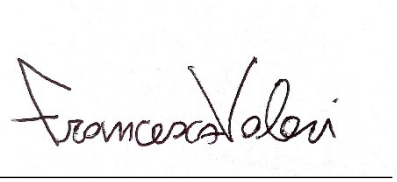

Francesco Valeri

Place and date: Rome (Italy), 24/04/2023

## Acknowledgements

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# Table of content

# I. List of abbreviations

| | |
|---|---|
| 129 | Mouse strain |
| 5XFAD – FMT | 5XFAD mice that received mock (PBS) treatment |
| 5XFAD + FMT young | 5XFAD mice that received cecal content from young donors (C57BL/6) |
| 5XFAD | Alzheimer's disease mouse model (Mutations: APP KM670/671NL (Swedish), APP I716V (Florida), APP V717I (London), PSEN1 M146L (A>C), PSEN1 L286V) |
| ABXs | Antibiotics' mixture |
| AChEIs | Acetylcholinesterase inhibitors |
| AD | Alzheimer's disease |
| ADAM | A Disintegrin And Metalloproteinase |
| ADL | Activities of daily living |
| $ADLP^{APT}$ | Alzheimer's disease mouse model (Mutations in three human transgenes, including amyloid precursor protein, presenilin-1, and tau, with six mutations) |
| AICD | APP intracellular domain |
| ANS | Autonomic nervous system |
| ApoA1 | Apolipoprotein A1 |
| APOE | Apolipoprotein E |
| APP | Amyloid precursor protein |
| APPswe/PS1ΔE9 | Alzheimer's disease mouse model (Mutations: APP KM670/671NL (Swedish), PSEN1: ΔE9) |
| Arc-sfGFP | Mouse model: ARC-creERT2/+.R26CAG-LSL-Sun1-sfGFP-Myc/+ |
| Arctic (E693G) | Alzheimer's disease mouse model (Mutations: located at codon 693 within the Aβ region of APP, at which glutamic acid is substituted for glycine) |
| ASD | Autism spectrum disorders |
| ASO | α-synuclein-overexpressing |
| Aβ | β-amyloid |
| BACE1 | β-secretase Beta-site APP cleaving enzyme 1 |
| BBB | Blood brain barrier |
| BDNF | Brain-derived neurotrophic factor |
| BMI | Body Mass Index |
| BSA | Bovine serum albumin (Albumin Fraktion V) |
| C3H | C3H/HeJ mouse strain |
| C57BL/6 | C57 black 6 mouse strain |
| C57BL/6N | C57 black 6 mouse strain |
| CA1 | Cornu Ammonis 1 |
| CAA | Cerebral amyloid angiopathy |
| $CaCl_2 \cdot 2H_2O$ | Calcium chloride dihydrate |
| CD-1 | Mouse model (deficient in both the *Cd1d1* and *Cd1d2* genes) |
| CDAD | *Clostridium difficile*-associated diarrhea |
| CDI | *Clostridium difficile* infection |
| CDR | Clinical Dementia Rating |
| CFUs | Colony forming units |
| CNS | Central nervous system |
| | Code: B6SJL-Tg (APPSwFlLon, PSEN1*M146L*L286V)6799Vas/Mmjax) |
| CSDS | Chronic social defeat stress |
| CSF | Cerebrospinal fluid |
| CTF | C-terminal fragment |
| CTRL | Control |
| DAPI | 4', 6-diamidino-2-phenylindole |
| DBA | Mouse strain |
| D-gal | D-galactose |
| dNTP | Deoxynucleotide |
| EDTA | Ethylenediaminetetraacetic acid disodium salt dihydrate |

| | |
|---|---|
| EFAD | Alzheimer's disease mouse model (5XFAD mice bred to homozygous APOE2-, APOE3-, and APOE4-TR mice: $5XFAD^{+/-}/APOE^{+/+}$) |
| ENS | Enteric nervous system |
| ER | Endoplasmic reticulum |
| Erα | Estrogen receptor α |
| FAD | Familial Alzheimer's disease |
| FCS | Fetal bovine serum |
| FDA | Food and Drug Administration |
| FMT | Fecal material transplant |
| FVB/N | Mouse strain |
| GBA | Gut-brain axis |
| gDNA | Genomic DNA |
| GFAP | Glial fibrillary acidic protein |
| GI | Gastrointestinal |
| GIT | Gastrointestinal tract |
| GOSs | Galacto-oligosaccharides |
| GVD | Granulovacuolar degeneration |
| hAPP | Human APP |
| HCl | Hydrogen chloride |
| HE | Hematoxylin and Eosin |
| High ABXs | High dosage |
| HPA | Hypothalamus-pituitary-adrenal |
| IBD | Inflammatory bowel disease |
| IL-6; IL-8 | Interleukin |
| Inos | Inducible nitric oxide synthase |
| ISCs | Intestinal stem cells |
| JAM | Junctional adhesion molecules |
| KCl | Potassium chloride |
| $KH_2PO_4$ | Potassium dihydrogenphosphate |
| LBP | LPS binding protein |
| LC | Locus coeruleus |
| *LcZ* | *Lactobacillus casei Zhang* |
| LMMP | Longitudinal muscle |
| Low ABXs | Low dosage |
| LPS | Lipopolysaccharides |
| LTD | Long-term depression |
| LTP | Long-term potentiation |
| MAP | Microtubule associated protein |
| $MgCl_2 \cdot 7H_2O$ | Magnesium chloride Heptahydrate |
| $MgCl_2$ | Magnesium chloride |
| $Na_2HPO_4$ | Disodium phosphate |
| $Na_3C_6H_5O_7$ | Sodium Citrate |
| NaCl | Sodium chloride |
| $NaH_2PO_4 \cdot 2H_2O$ | Sodium phosphate monobasic dihydrate |
| $NaHCO_3$ | Sodium bicarbonate |
| NaOH | Sodium hydroxide |
| NFTs | Neurofibrillary tangles |
| NMDA | N-methyl-D-aspartate |
| NMRI/Bom | Mouse model |
| NO | Nitric oxide |
| norNOHA | Arginase inhibitor N(ω)-hydroxy-nor-l-arginine |
| NOS1 | NO synthase type I |
| NSG | Next generation sequencing |
| OMO | Oligosaccharides from *Morinda officinalis* |
| P14-P21-P28 | Post-natal day 14/21 and 28 |

| | |
|---|---|
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase chain reaction |
| PD | Parkinson's disease |
| PEN | Presenilin enhancer |
| PET | Positron emission tomography |
| PFA | Paraformaldehyde |
| PHF | Paired helical filaments |
| PSD-95 | Postsynaptic density protein 95 |
| PSEN1 or 2 | Presenilin 1 or 2 |
| qPCR | Quantitative Real Time-PCR |
| RAGE | Receptor for advanced glycation end products |
| RAWM | Radial arm water maze |
| ROS | Reactive oxidative species |
| SAD | Sporadic Alzheimer's disease |
| SAMP8 | Senescence-accelerated mouse prone 8 |
| sAPPα | Soluble N-terminal fragment APPα |
| sAPPβ | Soluble peptide APPβ |
| SCFAs | Short-chain fatty acids |
| SD | Standard deviation |
| SF | Straight filaments |
| TBS | Tris buffered saline |
| Th1 | T helper type 1 |
| ThT | Thioflavin-T |
| TLRs | Toll-Like Receptors |
| TNF-α | Tumor necrosis factor α |
| TREM2 | Triggering receptor expressed on myeloid cells 2 |
| TrkB | Tropomyosin-related kinase B |
| vLPO | Ventrolateral preoptic nucleus preoptic nucleus |
| WT | Wild type |
| ZO | Zonula occludens |
| α-syn | α-synuclein |

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# IV. German Summary

Die Alzheimer-Krankheit ist weltweit eine der häufigsten Formen von Demenz bei älteren Menschen, wobei zwischen genetischer (familiärer) und umweltbedingter (sporadischer) Form unterschieden wird. Obwohl enorme Anstrengungen unternommen wurden, gibt es derzeit keine endgültige Heilung für diese progredierende Krankheit, abgesehen von einigen medizinischen Behandlungen, die die Symptome vorübergehend lindern können. Die Ätiologie der sporadischen Form der Alzheimer-Krankheit ist komplex, da mehrere Faktoren eine Rolle spielen und die zugrunde liegenden Ursachen nicht vollständig verstanden oder identifiziert sind. In den letzten zehn Jahren hat sich die Rolle der Darmmikrobiota im Zusammenhang mit neurodegenerativen Erkrankungen (z. B. Morbus Parkinson und M. Alzheimer) herauskristallisiert, da sie über die Darm-Hirn-Achse die Gehirnfunktion und das Verhalten des Wirts regulieren kann. Daher wurden mehrere Studien an keimfreien Mäusen oder an Mäusen, die Antibiotika oder Probiotika erhielten, durchgeführt. Unter den auf der Mikrobiota basierenden therapeutischen Ansätzen wird die Transplantation der fäkalen Mikrobiota als mögliche therapeutische Option für die Alzheimer-Krankheit angesehen. Darüber hinaus ist das Altern der Hauptrisikofaktor für die Alzheimer-Krankheit und eine interessante Frage, die im Zusammenhang mit der Darmmikrobiota zu untersuchen ist.

In dieser Arbeit wurden mit Antibiotika vorbehandelte transgene 5XFAD-Mäuse (Modell für die Alzheimer-Krankheit) akut mit fäkaler Lösung behandelt, die aus Zäkummaterial von altersgleichen (1 Monat) oder mittelalten (1 Jahr) Wildtyp-Mäusen gewonnen wurde. Anschließend wurden physiologische Parameter (z. B. Körpertemperatur), Darmmorphologie, Verhalten sowie Alzheimer- und altersbedingte Merkmale (z. B. Amyloidablagerungen und LBP) untersucht. Die kultivierbaren fäkalen Keime der Enterobacteriaceae- und Lactobacillaceae-Familien wurden als Referenzbakterien verwendet, da sie eine wichtige gegensätzliche Rolle für die Homöostase des Wirts spielen. Zunächst wurden bei mittelalten ("alten", 1 Jahr) Spendern im Vergleich zu "jungen" (1 Monat alten) Wildtypspendern höhere Konzentrationen von Enterobacteriaceae und Lactobacillaceae festgestellt. Die chronische Verabreichung von Antibiotika konnte die Ausgangswerte für Enterobacteriaceae und Lactobacillaceae im Darm sowohl der Mütter der Empfängertiere als auch der 5XFAD-Nachkommen (Empfänger) reduzieren. Anschließend wurden die Enterobacteriaceae- und Lactobacillaceae-Konzentrationen in den 5XFAD-Empfängermäusen 1 und 6 Wochen nach der Verabreichung der Fäkalienlösung von Wildtyp-Spendern untersucht. Interessanterweise wurden zu beiden Zeitpunkten signifikant höhere Lactobacillaceae-Konzentrationen festgestellt, was darauf hindeutet, dass ein einmaliger, akuter Transfer von Zäkummaterial von älteren Spendern in der Lage war, zumindest teilweise einen gealterten Phänotyp bei den Empfängertieren zu etablieren. Als weitere Analyse bestätigte die qPCR eine verringerte Menge an Firmicutes sowohl bei den älteren Spendern als auch bei den mit Zäkuminhalt von alten Spendern behandelten 5XFAD-Mäusen.

Verhaltenstests, mit denen Gedächtnisverluste sowie Angst- und Depressionsähnliche Verhaltensweisen, die mit der AD-Pathologie in Verbindung gebracht werden, beurteilt werden (d. h. *Nesting*-Test, Radialarm-Wasserlabyrinth, T-Labyrinth und Neophobie-Test), zeigten keine Unterschiede aufgrund der erhaltenen Behandlungen, was auf das vergleichsweise junge Alter der Empfängertiere zurückzuführen sein könnte. Einige der untersuchten Duodenalstrukturen (d.h. Zottenlänge, Submukosa- und Muskeldicke), von denen bekannt ist, dass sie eine entscheidende Funktion bei den Absorptions- und Stoffwechselprozessen ausüben, wiesen bei 5XFAD-Mäusen, die Zäkuminhalt von alten Spendern erhielten, eine deutlich veränderte Morphologie auf. Zu den physiologischen Merkmalen, die mit dem Alterungsprozess in Verbindung gebracht werden, gehört eine niedrigere Körpertemperatur bei den Empfängern von altem Spendermaterial. Dies und ein erhöhter Spiegel des Lipopolysaccharid-Bindungsproteins im Serum (LBP), eines Entzündungsproteins, das mit der Darmpermeabilität bei Erwachsenen und der Darmmikrobiota in Verbindung gebracht wird, deutet auf eine mögliche Übertragung von altersbedingten Merkmalen bei den Empfängermäusen hin. Darüber hinaus zeigten Analysen einiger mit der Alzheimer-Krankheit assoziierter Hirnregionen (z. B. präfrontaler Kortex und Gyrus dentatus) eine höhere Plaque-Belastung bei 5XFAD-Mäusen nach dem Transfer von Material älterer Tiere, was auf eine Verschlimmerung der mit der Transplantation verbundenen Pathologie schließen lässt.

Zusammenfassend lässt sich sagen, dass gesunde Wildtypspender in der Lage waren, einige mit der Alterung verbundene Merkmale in einem Mausmodell für die Alzheimer-Krankheit zu übertragen und letztlich die frühen pathologischen Merkmale negativ zu beeinflussen.
Frühere Berichte haben gezeigt, dass die Gefahr besteht, dass Pathologien, die mit der Darmmikrobiota in Verbindung stehen, wie Diabetes oder Fettleibigkeit, auf die Empfängermäuse übertragen werden. Unter den Umweltfaktoren, von denen bekannt ist, dass sie die Pathogenese der sporadischen Alzheimer-Krankheit beeinflussen, wurde in einer Vielzahl von Tier- und Humanstudien eine wiederholte Belastung durch Stress festgestellt. In dieser Arbeit wurde die Rolle von Stress in Bezug auf die Aktivierung der enterischen Neuronen bei Tamoxifen-induzierten Arc-sfGFP-Mäusen (im Alter von 4 Wochen) untersucht, die einem chronischen Stressparadigma der sozialen Niederlage ausgesetzt waren. Interessanterweise zeigten die Mäuse, die dem Stressparadigma ausgesetzt waren, eine signifikant höhere Anzahl GFP-positiver Neuronen im myenterischen Plexus des Ileums im Vergleich zu Kontrollmäusen, die keinem Stress durch chronische soziale Niederlage ausgesetzt waren. Weitere Studien sind erforderlich, um die Mechanismen zu erforschen, die dem Zusammenhang zwischen Stressereignissen und der Aktivierung der enteralen Neuronen zugrunde liegen, und um herauszufinden, ob das Mikrobiom in diesem Paradigma eine aktive Rolle spielt oder durch den Stress ebenfalls maßgeblich beeinflusst wird.

# V. English Summary (Abstract)

Alzheimer's disease represents one of the most common forms of dementia among the elderly worldwide divided into genetic (familial) and environmental (sporadic) forms. Although enormous effort has been carried out, currently there is not a definitive cure for this debilitating disease aside from some medical treatments that can temporarily reduce the symptoms. The etiology of Alzheimer's disease sporadic form is complex as several factors have been involved and the underlying causes are not completely understood or identified. In the last decade, the role of the gut microbiota has been emerged in relation to neurodegenerative disorders (e.g., Parkinson's and Alzheimer's diseases) as it regulates the host brain function and behavior through the gut-brain axis. Therefore, several studies have been conducted on germ-free mice or mice subjected to antibiotics or probiotics. Among the microbiota-based therapeutic approaches, the fecal microbiota transplant has been considered as a valid therapeutic option for Alzheimer's disease. In addition, aging is the main risk factor for Alzheimer's disease and an intriguing question to address in relation to the gut microbiota.

In this thesis, antibiotics-pretreated transgenic 5XFAD mice for Alzheimer's disease were acutely administrated with fecal solution provided by cecal material of age-matched (1-month-old, "young") or middle aged (12-months-old, "old") wild type mice. Then, physiological parameters (e.g., body temperature), gut morphology, behavioral tasks, and Alzheimer's disease and aging related hallmarks (e.g., amyloid deposition and LBP) were addressed. Cultivatable fecal Enterobacteriaceae and Lactobacillaceae families were used as reference bacteria since they exert a main opposite role on the host homeostasis. Firstly, higher levels of Enterobacteriaceae and Lactobacillaceae were assessed in middle aged compared to young wild type donors. Chronic antibiotics administration was able to reduce the baseline Enterobacteriaceae and Lactobacillaceae levels in the gut of both, dams and 5XFAD offspring mice. Then, Enterobacteriaceae and Lactobacillaceae levels were monitored in recipient 5XFAD mice at 1 and 6 weeks after having received fecal solution from wild type donors. Interestingly, significantly higher Lactobacillaceae levels were detected at both set time points, suggesting that a single-time, acute transfer of cecal material from the older donors was able to establish, at least partially, an aged phenotype in the recipient animals. As a further analysis, qPCR confirmed a decreased amount of Firmicutes in both donors' and in 5XFAD mice treated with cecal content from old donors.

Behavioral tests, used to assess memory decline as well as anxiety- and depressive-like behaviors associated to AD pathology (i.e., nesting test, radial arm water maze, T-maze and neophobia test), revealed lack of differences due to the received treatments, which might be due to the comparably young age of the recipient animals. Some of the investigated duodenum structures (i.e., villus length, submucosa and muscularis thickness), known to exerts a crucial function in the absorption and metabolism processes, showed significantly altered morphology in 5XFAD mice that received cecal content from old donors. Among the physiological features associated to the aging process, recipients of old donor material displayed lower body temperature. This, together with an increased level of serum lipopolysaccharide binding protein, an inflammation protein associated to intestinal permeability in adults and to gut microbiota, indicates a possible transmission of aging-related features in recipient mice. In addition, analyses of some of the brain regions (i.e., prefrontal cortex and dentate gyrus) associated to Alzheimer's disease showed higher plaque load in 5XFAD mice after transfer of material from older animals, suggesting a worsening of the pathology associated to the transplantation.

In conclusion, healthy wild type donors were able to transmit some features associated to aging in a mouse model for Alzheimer's disease and ultimately affected its early pathological hallmarks. Previous reports revealed the risks of transmitting pathologies associated to gut microbiota, such as diabetes or obesity, into the recipient mice. Therefore, anatomic and environmental differences between humans and mouse models need to be considered in experimental design. Among the environmental factors known to influence the pathogenesis in sporadic Alzheimer's disease, repeated exposure to stress has been reported in a wide variety of animal and human studies. Finally, in this thesis, the role of stress has been investigated in relation to enteric neurons activation in Tamoxifen-induced Arc-sfGFP mice (aged 4 weeks) subjected to chronic social defeat stress paradigm. Interestingly, mice subjected to stress paradigm showed significantly higher GFP-positive neurons cell counts in the ileum intestine *myenteric plexus* as compared to control mice that were not exposed to chronic social defeat. Further studies are

required to shed light on the mechanisms underlying the connection between stressful events and the enteric neuron activation and if the microbiome plays an active role in this paradigm.

# 1. Introduction

## 1.1 Alzheimer's disease

Alzheimer's disease (AD) is one of the most common neurodegenerative disorders and one of the most common forms of dementia, affecting more than 35 million of people worldwide with an incidence over 50% of all dementia cases (Prince, 2015). The symptoms were described for the first time by Alois Alzheimer in a 52-year-old woman, Auguste Deter, who was his patient at the state asylum of Frankfurt in Germany (Stelzmann et al., 1995). Very impressive was the neuropsychological characterization of the disease by Alzheimer (Alzheimer, 1907; for an English translation see Stelzmann et al., 1995):

*"Her memory is seriously impaired. If objects are shown to her, she names them correctly, but almost immediately afterwards she has forgotten everything. When reading a test, she skips from line to line or reads by spelling the words individually, or by making them meaningless through her pronunciation. In writing she repeats separate syllables many times, omits others and quickly breaks down completely. In speaking, she uses gap-fills and a few paraphrased expressions ("milk-pourer" instead of cup); sometimes it is obvious she cannot go on. Plainly, she does not understand certain questions. She does not remember the use of some objects."*

Nowadays, there is more information about AD pathology. However, the cardinal features required for the assessment of the pathological diagnosis are still the ones that were firstly described by Alzheimer himself. Indeed, to date, AD is characterized by the presence of extracellular deposition of β-amyloid (Aβ) in the form of diffuse senile or neuritic plaques and by intraneuronal neurofibrillary tangles (NFTs) and neuropil threads.
Concerning the symptoms, AD patients are characterized by a premature impairment in learning and memory and by impairments in attention, executive function, language, visuospatial function, and social behavior at a later stage of the disease (McKhann et al., 2011). This will result inevitably in death generally within 5–12 years from the onset of the symptoms (Vermunt et al., 2019). Clinical Dementia Rating (CDR) can be used to classify the severity of the dementia basing on composite level of dysfunction in the areas of memory, orientation, judgement, and problem solving, involvement in community affairs, function in home and hobbies, and self-care (Burke et al., 1988; Long and Holtzman, 2019; Morris, 1997). The *ante-mortem* diagnosis is validated with cerebrospinal fluid (CSF) or positron emission tomography (PET) imaging biomarkers (Brier et al., 2016; Fagan et al., 2006; Lowe et al., 2019; Morris et al., 2009).
The age of the population represents the main factor to consider when analyzing the incidence of AD; the incidence in the population over 65 years of age raises from 1-5% to 20-25% in the population over 80 years old (Bekris et al., 2010; Brickell et al., 2006).

## 1.2 The familial and sporadic forms of Alzheimer's disease

AD may target different members of the same family (Amaducci et al., 1986; Farrer et al., 1989; Fitch et al., 1988; Heston et al., 1981) due to genetic transmission (Goate et al., 1991; Schellenberg et al., 1992; St. George-Hyslop et al., 1987). In addition, the observation of diverse phenotypic features of AD segregated according to the family origin raised the possibility of several distinct genotypes (Bird et al., 1989; Duara et al., 1993). However, AD patients may not necessarily be affected by the genetic (familial, FAD) form but also by a sporadic AD (SAD) form which is etiologically heterogeneous and results from a combination of many genetic and environmental factors – some of them probably still not identified. The SAD represents the most prevalent form that occurs in people over 65 years of age, while only 1-5% of AD cases are represented by FAD, which occurs in people before 65 years of age (Alzheimer Association, 2019).
FAD is caused by genetic mutation of several genes, such as the *amyloid precursor protein* (*APP*), *presenilin 1* (*PSEN1*) or *presenilin 2* (*PSEN2*) that are involved in the pathways for amyloid production (Sorbi et al., 2001; Tanzi and Bertram, 2005; Żekanowski and Wojda, 2009). The SAD form seems to be caused by molecular changes, including methylation, oxidative damage in certain genes, and by

dysregulation in the calcium homeostasis (Lahiri et al., 2007). In particular, 70% of SAD cases are caused by genetic risk factors, while the other 30% are caused by environmental exposition, such as diet, toxic substances, and hormonal factors, but also by stress experiences (Alonso Vilatela et al., 2012; Bisht et al., 2018; Jones et al., 2015; Justice, 2018; Lahiri et al., 2007). Such molecular changes together with the absence of a fully functional repair system may be the underlying cause for the appearance of the AD symptoms (Lahiri et al., 2007). In addition, these two forms seem to have a different molecular basis that may differently impact the experimental therapy and diagnosis to adopt (Dorszewska et al., 2016).

## 1.3 Risk factors in Alzheimer's disease: a focus on aging and sex

Genetics play a strong role in AD for both FAD and SAD forms. In particular, *APP* and *PSEN1/2* genes have been correlated only with the FAD form, while *apolipoprotein E* (*APOE*) and *triggering receptor expressed on myeloid cells 2* (*TREM2*) genes correlated with the SAD form (Corder et al., 1993; Fryer et al., 2003; Leduc et al., 2010; Pottier et al., 2013). A more detailed explanation of the mechanisms behind genetic risk factors will be given in the next paragraphs (see subparagraphs 1.4.2.1 and 1.4.2.2). However, aside genetics, also demographic factors, lifestyle, infections, and environment are also involved in the development of AD pathology (Table 1). Among the demographic factors, age is considered the most important risk factor for cognitive decline and AD (Doruk et al., 2010; Herrup, 2010). In particular, the prevalence of AD increases to 19% in individuals with an age between 75-84 years and to 30-35% for the ones older than 85 years (Ferri et al., 2005; Knopman, 2001). Some studies support the idea that AD could be an accelerated form of normal aging because many of the pathological changes identified in AD are similar to those described in normal aging (Rossor and Mountjoy, 1986). Examples of such similar aspects may be represented by reduction in brain volume and weight, enlargement of ventricles, and loss of synapses and dendrites (Imhof et al., 2007). As a matter of fact, Dewolfe Miller et al. (1984) conducted an observation on 199 cognitively normal elder individuals (from 71 years of age or older) showing that 32/60 had no senile plaques, 13/60 had senile plaques in the hippocampus, and 12/60 had senile plaques in temporal cortex (Dewolfe Miller et al., 1984). The authors concluded the impossibility of distinguishing early AD from normal aging by *post-mortem* investigation due to the common macroscopic features (Dewolfe Miller et al., 1984). In addition, another study showed that the density of senile plaques with a distinct core ("classic" plaques) were not significantly different between AD patients and normal elders (Bell and Ball, 1990). However, opposite results have been shown in relation to the presence of NFTs between normal aged brain and AD patients' brain (Bos et al., 2017; Coria et al., 1993; Knopman et al., 2003). In particular, two studies suggested that most of cognitively normal individuals present low NFTs density in the brain (Coria et al., 1993; Knopman et al., 2003), while another study showed that high NFTs density is present in the layer II of the entorhinal cortex and in the Cornu Ammonis (CA1) region of hippocampus of all non-demented individuals (Bos et al., 2017). Guillozet et al. (2003) suggested that the high presence of NFTs in non-demented individuals' brain may be associated with normal memory loss (Guillozet et al., 2003). Nevertheless, AD patients' brains are characterized by higher sensitivity in the development of NFTs in the brain perforant path, a connectional route between the entorhinal cortex and all CA fields of the hippocampus, compared to non-diseased aged people (García-Sierra et al., 2000). Difficulties in discriminating physiologic aging from AD are also due to two shared common features, such as breakdown of myelin together with subsequent breakdown of white matter fiber tracts, and loss of cells in the brain stem nuclei (e.g., in the locus coeruleus (LC)) (Armstrong, 2019; Bartzokis, 2011). The aging process impacts several systems leading to glucose hypometabolism, mitochondria dysfunction, innate immune and inflammatory reaction, amyloid production, dysregulation of cholesterol homeostasis, white matter degeneration, and decline in regenerative capacity; all of them contributing to AD development (Riedel et al., 2016).

Sex is another important variable to consider when assessing the AD risk. Historically, sex was the first factor considered when assessing AD risk because about two thirds of persons that were diagnosed with AD were women (Alzheimer's Association, 2017). However, the statement "women are at the greater risk" needs to be reconsidered since life expectancy of women is, in general, longer than that of men and age is the greatest risk factor for AD (Mielke, 2018). However, taking into consideration male and female patients at the same age, lifetime risk of getting AD is indeed greater in women (Plassman et al., 2007). To date, it is still not clear why women are more prone to develop AD compared to men. This may also

depend from different countries and distinct time periods (Mielke, 2018; Rocca, 2017). In addition, AD mechanism pathways and risk factors may differently impact men and women. Several hypotheses have been made by which sex may differently affect the risk of developing AD (Mielke, 2018). On one hand, a stronger effect in one of the two sexes may be due to risk factors occurring with the same frequency in both women and men (e.g., autosomal chromosomes present *APOE* genotype) (Mielke, 2018; Rocca et al., 2014). On the other hand, risk factors with the same effect in both, women and men, may occur with different frequency (e.g., less access to education for women or higher prevalence of smoking for men). Other risk factors may differ for frequency and effects by sex (e.g., head trauma is more common in men as compared to women, but higher susceptibility to adverse effects has been observed in women) or might be delimited by sex differences (e.g., pregnancy, oophorectomy; prostate cancer, and androgen deprivation therapy) (Mielke, 2018; Rocca et al., 2014). In the last decade, several studies showed that the gut microbiota varies according to sex differences and age (for a review see Valeri and Endres, 2021), and therefore might provide an additional factor to be considered in relation to AD. A detailed explanation of sex differences in relation to the gut microbiome and AD is reported in chapter 1.7.3.

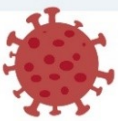
| Demographic | *References* |
|---|---|
| Age | Doruk et al., 2010 |
| | Herrup, 2010 |
| | Henderson, 2010 |
| | Knopman, 2001 |
| | Ferri et al., 2005 |
| Sex | Henderson, 2010 |
| | Plassman et al., 2007 |
| | Rocca, 2014 |
| Education | Henderson, 2010 |
| | Jonaitis et al., 2013 |
| | Gaillard, 1984 |
| Social class | Henderson, 2010 |

| Lifestyle | *References* |
|---|---|
| Alcohol | Grant, 2014 |
| Lack of physical activity | Henderson, 2010 |
| | Kirk-Sanchez et al., 2014 |
| | Rantanen, 2013 |
| Malnutrition/Poor diet | Abalan, 1984 |
| | Henderson, 2010 |
| | Von Arnim et al., 2010 |
| | Scarmeas et al., 2006 |
| | Schreurs et al., 2013 |
| | Wang et al., 2010 |
| Smoking | Chang et al., 2014 |
| | Henderson, 2010 |

| Enviroment | *References* |
|---|---|
| Air pollution | Killin et al., 2016 |
| Geographic location | Henderson, 2010 |
| Metals | Arsene et al., 2011 |
| | Hsu et al., 2018 |
| | Peters et al., 2013 |
| Organic solvents | Henderson, 2010 |
| Military service | Goldstein et al., 2012 |
| Occupation | Killin et al., 2016 |
| | Peters et al., 2013 |
| Vitamin deficency | Grimm et al., 2016 |
| | McCaddon and Kelly, 1994 |
| | Mortimer et al., 1985 |

| Genetics | *References* |
|---|---|
| Amyloid precursor protein (*APP*) | Charier-Harlin, 1991 |
| | Goate, 1991 |
| Presenilin 1 and 2 (PSEN1/2) | Levy-Lahad et al., 1995 |
| | Sherrington et al., 1993 |
| ATP-binding cassette transporter A1 (ABCA1) | Koldamova et al., 2010 |
| Adaptor protein evolutionarily conserved signalling intermediate in Toll pathway (ECSIT) | Soler-Lopez et al., 2012 |
| Clusterin gene (CLU) | Ponomareva et al., 2013 |
| Fermitin family homolog 2 gene (FERMT2) | Chapuis et al., 2017 |
| Estrogen receptor gene (ESR) | Ryan et al., 2014 |
| Histocompatibility locus antigen (HLA class III) | Cohen et al., 1982 |
| | Henschke et al., 1978 |
| | Walford and Fortoul, 1983 |
| Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | Allen et al., 2012 |
| Triggering receptor expressed on myeloid cells 2 (TREM 2) | Ruiz et al., 2014 |
| | Corder et al., 1993 |
| | Strittmatter et al., 1993 |
| | Pottier et al., 2013 |
| | Chartier-Harlin et al., 1991 |
| | Fryer et al., 2003 |

| Medical | *References* |
|---|---|
| Cancer | Soininen t al., 1982 |
| Cardiovascular disease | Kalaria, 2010 |
| | Liu et al., 2014 |
| Obesity | Bouras et al., 1993 |
| | Mazon et al., 2017 |
| Diabetes | Akter et al., 2011 |
| | Luchsinger, 2009 |
| Stroke | Zhang and Le, 2010 |
| Infarts | Benedictus et al., 2013 |
| Cholesterol | Leduc et al., 2010 |
| Immune system dysfunction | Armstrong et al., 1995 |
| TBI | Geddes et al., 1999 |
| | Heyman et al., 1984 |
| | McKee et al., 2013 |
| | McKee et al., 2014 |
| Depression | French et al., 1985 |
| | Barclay et al., 1986 |
| Early stress | Hoeijmakers et al., 2017 |
| | Piirainen et al., 2017 |



| Infection | *References* |
|---|---|
| Bacteria | Balin et al., 1998 |
| | Gérard et al., 2006 |
| | Miklossy, 2015 |
| Dental infection | Rolim et al., 2014 |
| Fungi | Alonso et al., 2014 |
| Viruses | Libikova et al., 1978 |
| | Salazar et al., 1983 |
| | Wisniewski et al., 1981 |

**Table 1: Common risk factors associated to sporadic Alzheimer's disease.** The reported information was selected taking into account original works and current reviews on the risk factors associated to Alzheimer's disease (i.e., Armstrong, 2019; Caruso et al., 2019; Zhang et al., 2022).

## 1.4 Brain histology and molecular basis of Alzheimer's disease

Brains of AD patients are characterized by neuropathological lesions that involve the massive presence of Aβ plaques, NFTs, neuropil threads and dystrophic neurites (Crews and Masliah, 2010; Iqbal and Grundke-Iqbal, 2002; Terry et al., 1994; Trojanowski and Lee, 2000). These changes are frequently associated with astrogliosis (Beach et al., 1989; Itagaki et al., 1989) and microglial cell activation (Itagaki et al., 1989; Masliah et al., 1991; Rogers et al., 1988). In addition, some AD brain areas present unique lesions (e.g., Hirano bodies and granulovacuolar degeneration in the hippocampus) and loss of neurons, neuropil, and synaptic elements (DeKosky and Scheff, 1990; Gómez-Isla et al., 1997; Knowles et al., 1999; Masliah et al., 1991; Scheff et al., 2007, 1990; Scheff and Price, 1993). Such lesions are distributed in the brain with amyloid plaques taking place throughout the cerebral cortex and NFTs occurring primarily in limbic and association cortices (Arnold et al., 1991; Braak and Braak, 1991; Thal et al., 2002). Several studies observed that neuronal and synapse loss are typical events that accompany NFTs formation. This suggests that NFTs may be the cause of such lesions in the brain (De Calignon et al., 2010; Gómez-Isla et al., 1997; Iqbal and Grundke-Iqbal, 2002; Kimura et al., 2010; Spires-Jones et al., 2008). A consistent hierarchical pattern of degeneration among brain regions has been accepted as part of the 1997 NIA-Reagan diagnostic criteria (NIA-RI Consensus 1997) and describes the presence of a staging scheme. In particular, the entorhinal perirhinal cortex is considered as the first region to be affected, followed by the hippocampal CA subdomains and association cortex. Finally, the primary neocortex is the last brain region to be impacted (Serrano-Pozo et al., 2011). To date, the best correlate for the cognitive impairment of AD patients is represented by progressive neurodegeneration (synapses impairment together with axon degeneration and consequent dendritic tree atrophy) in the limbic system, neocortex and basal forebrain (Arnold et al., 1991; DeKosky et al., 1996; DeKosky and Scheff, 1990; Klucken et al., 2003; Masliah et al., 1991; Teipel et al., 2005; Terry et al., 1994). Aside senile plaques and NFTs, several investigations support the idea that increased levels of soluble Aβ1–42 oligomers might lead to synaptic damage and neurodegeneration (Glabe, 2005; Lacor et al., 2007; Townsend et al., 2006; Walsh and Selkoe, 2005) and might be responsible for the AD-related memory deficits (Lacor et al., 2007). Indeed, structural changes in the brain of AD patients are normally preceded by amyloid deposition and tau hyperphosphorylation (review Jack et al., 1999). These molecular changes start to appear already more than two decades before the onset of AD dementia (Fortea et al., 2020). A more detailed description of macroscopic and microscopic features in AD brain patients is reported in the next sub chapters (1.4.1 and 1.4.2).

### 1.4.1 Macroscopic features

Macroscopic examination of AD patients' brains by *post-mortem* analysis revealed no specific single features or mixture of features that can be explicitly considered diagnostic. Indeed, some common changes in AD brains are also presented in the brains of elderly individuals who had displayed normal cognitive function during life (review Perl, 2010). In particular, AD brains revealed some common features, including symmetric pattern of cortical atrophy predominantly affecting the medial temporal lobes and relatively sparing the primary motor, sensory and visual cortices (Serrano-Pozo et al., 2011). Moreover, atrophy in the brain cortex induces a prominent dilation of the lateral ventricles together with the symmetrical expansion of the *ex-vacuo* hydrocephalus. Such features are considered stereotypic and can be recognized early in the clinical course of the disease by MRI scan (Dickerson et al., 2011, 2009). In detail, AD brains are characterized by enlarged sulcal spaces with atrophy of the gyri in the frontal and temporal cortices, while lack of alteration was observed in the somatosensory and motor cortices (review Perl, 2010). Functional imaging studies regarding AD revealed increased atrophy in the posterior cortical areas at the precuneus and posterior cingulate gyrus level (Rami et al., 2012; Zhou et al., 2010).
Despite these common macroscopic features in AD patients, aged, clinically unaffected people may show similar characteristics, such as moderate cortical atrophy in the frontal lobes and volume loss in white matter (Piguet et al., 2009). A comparison between brain and relative neuron structure in cognitively healthy people and in AD patients is reported in figure 1.

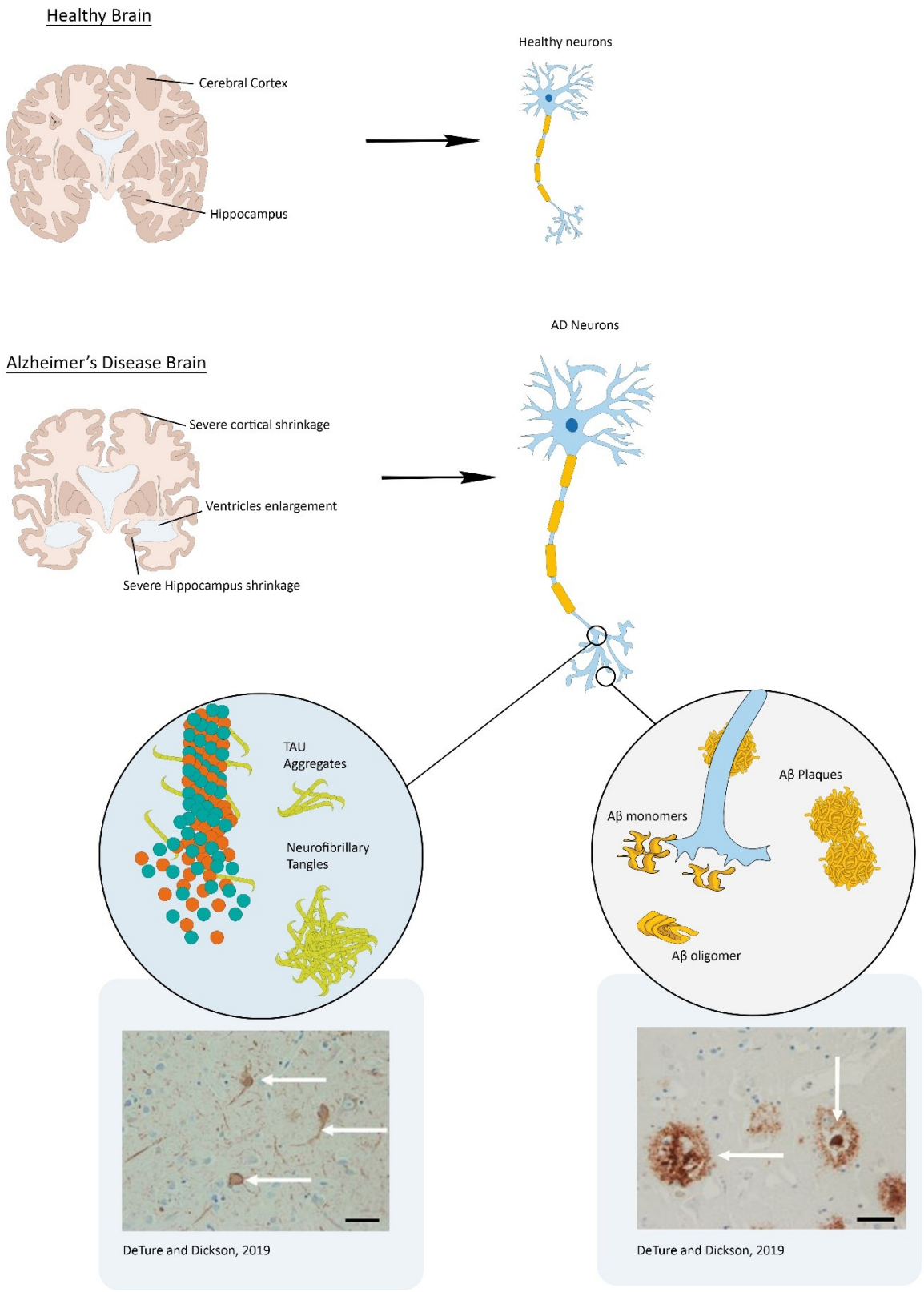



**Figure 1: Brain and relative neuron structure in cognitively healthy people and in AD patients.** Severely affected AD patients present common features, such as shrinkage in cortical and hippocampus areas and ventricle enlargement. AD neurons are characterized by pathological features, including aggregation of hyperphosphorylated tau proteins into NFTs along the neuronal axon and by Aβ plaque deposition in the brain (picture below, white arrows indicate the respective structures). Brain histological examples of NFTs and senile plaques were obtained from Deture and Dickson (2019). Abbreviations: AD (Alzheimer's disease), Aβ (β-amyloid).

In addition, atrophies in the amygdala and hippocampus together with temporal horn enlargement are common features in AD patients (Apostolova et al., 2012; Perl, 2010; Serrano-Pozo et al., 2011) but also of other age-related disorders (e.g., hippocampal sclerosis or argyrophilic grain disease) (Deture and Dickson, 2019). Such macroscopic brain observations are not specific for AD but are extremely supportive due to lack of brain microscopic examinations.
Among other conditions that frequently accompany aging and AD, chronic hypertension and vascular-related diseases represent high risk factors and possible indicators for AD pathology (Serrano-Pozo et al., 2011). Suspicious severe cerebral amyloid angiopathy is responsible for AD and may be caused by cortical petechial microbleeds, cortical microinfarcts or even evident lobar hemorrhages in the posterior parietal and occipital lobes, lacunar infarcts in the basal ganglia and by demyelination of the periventricular white matter (Serrano-Pozo et al., 2011).

### 1.4.2 Microscopic features

The microscopic examination of multiple brain regions using staining methods represents the final tool for detecting AD neuropathological changes (Montine et al., 2012). AD shares some common features with other neurodegenerative diseases that contributes to the idea of a mixed pathology (Deture and Dickson, 2019). However, as already described 100 years ago (reviewed by Ryan et al., 2014), the presence of Aβ plaques and NFTs in the brain are the most common features for AD diagnosis (Fig. 1). Other common diagnostic features are represented by tau-positive neuropil threads, dystrophic neurites, activated microglia, and reactive astrocytes as well as eosinophilic Hirano bodies, granulovacuolar degeneration (GVD), and cerebral amyloid angiopathy (CAA) (Perl, 2010; Serrano-Pozo et al., 2011). Altogether, such hallmarks induce loss of synapses and neurons in vulnerable brain regions responsible for symptoms generally connected with AD (Perl, 2010; Serrano-Pozo et al., 2011).

#### *1.4.2.1 Neurofibrillary tangles and tau proteins*

The inclusions within the perikaryal cytoplasm of pyramidal neurons of AD patients' brains are called NFTs. Their distribution in AD patients' brains includes the CA1 and subicular regions of the hippocampus, the entorhinal cortex the layer II, the amygdala, and the neocortex deeper layers (e.g., layers III, V, and superficial VI) (Morrison and Hof, 1997). In particular, several studies correlate the degree of dementia caused by AD and the duration of illness with the extent and distribution of NFTs (Arriagada et al., 1992; Bierer et al., 1995), suggesting a direct effect of these tangles on the functionality of the brain.
At structural level, NFTs consist of bundles of aggregates of abnormally, hyperphosphorylated filaments of tau proteins that are polymerized into paired helical filaments (PHF) and mixed with straight filaments (SF) (Grundke-Iqbal et al., 1986; Iqbal et al., 2010). While NFTs accumulate in the neuronal cell body, neuropil threads consist of abnormally phosphorylated straight and paired helical tau filaments located in distal dendrites (Perry et al., 1991).
Tau is a microtubule-associated protein (MAP) that presents six isoforms encoded by a single tau gene on the chromosome 17 generated by alternative splicing of its pre-mRNA (Himmler et al., 1989; Iqbal et al., 2010). In particular, such isoforms may differently affect the microtubule stability (Kopke et al., 1993; Lindwall and Cole, 1984). In physiologic condition, tau proteins exert their function in the brain by stabilizing microtubules and promoting the assembly of tubulins (Weingarten et al., 1975). In AD, tau proteins are subjected to hyperphosphorylation and to subsequent abnormal folding compared to unassembled normal tau (Alonso et al., 1994). Many cellular processes have been involved in tau phosphorylation state (e.g., GSK3β, MAPK, CK1δ and Cdk5 kinases), and in tau dephosphorylation state (e.g., PP2A phosphatase) (Brunello et al., 2020). Thus, an imbalance in the upregulation of the kinases' activity and in the downregulation in the phosphatase' activity may be the cause of the generation of the abnormal hyperphosphorylated tau (Brunello et al., 2020). Several evidences suggested that the pathological hyperphosphorylation may depend on different types of tau phosphorylation sites or on different forms of tau (Brunello et al., 2020; Schneider et al., 1999). However, tau hyperphosphorylation mechanism is rather complicated and its main causes are still unclear.

### *1.4.2.2 Senile plaques and β-amyloid production*

Senile amyloid plaques are generated by the extracellular accumulation of the peptides Aβ (its length ranges between 37 and 43 amino acids units), which result from the sequential aberrant cleavage of the APP by β- and γ-secretase, respectively (Goedert, 2009; Kumar et al., 2015; Thal et al., 2006). Aβ consist of a small 4 KDa peptide that fold into a β-pleated sheet configuration and constitute the central core of the senile plaques, while the surrounding abnormally formed neurites generate the corona of the senile plaques (Kang et al., 1987; Masters et al., 1985). Several protein components have been associated to the central core of the senile plaques, such as heparan sulfate glycoproteins, complement proteins, APOE and α-1-antichymotrypsin (Abraham et al., 1990; Castillo and Snow, 1996; Dickson and Rogers, 1992). The external region of neuritic plaques is mostly populated by microglial cells and, less frequently, by reactive astrocytes (Perl, 2010). Due to the higher amount of β-pleated sheet configuration of the Aβ peptide, senile plaques can bind the planar dye Congo red and generate a birefringence signal when induced by polarized light (Sipe and Cohen, 2000). Moreover, amyloid fibrils can also be visualized by thioflavin T (ThT), a dye that, when excited at 450 nm, produces a high fluorescent signal around 482 nm (Naiki et al., 1989; Wu et al., 2008). The Aβ peptides tend to accumulate also in the cortical blood vessel walls (e.g., in the small arteries and arterioles of the leptomeninges) of the brain aside from the central core of neuritic plaques, creating a lesion called "congophilic angiopathy" because it can be visualized by Congo red (Perl, 2010). The majority of brain areas in which the hemorrhages tend to occur are the frontal or occipital poles in the form of small and multiple lesions, while larger lesions are frequently called "lobar hemorrhages" and represent one of the fatal complications in AD (Perl, 2010).

APP is a transmembrane protein that can be subjected to an amyloidogenic or to a non-amyloidogenic pathway (Fig. 2). In the non-amyloidogenic pathway, which is thought to have a protective effect against the AD pathology antagonizing the Aβ production, APP is cleaved by the α-secretase generating two non-toxic fragments, such as a soluble N-terminal fragment APP-α (sAPPα) and a C-terminal fragment (CTF) CTF83. This process is also mediated by one or more enzymes from the family of A Disintegrin And Metalloproteinase (ADAM), including ADAM 9, 10, 17 and 19 (Asai et al., 2003; Sahlin et al., 2007; Tanabe et al., 2007). The physiologic function of sAPPα is poorly understood but, several studies showed its beneficial effect on neurons, including protection against oxygen-glucose deprivation and excitotoxicity, stabilization of membrane potential (Furukawa et al., 1999; Mattson et al., 1993), promotion of neurite outgrowth, synaptogenesis and cell adhesion (Gakhar-Koppole et al., 2008; Mattson, 1997)(Fig. 2B).

In the amyloidogenic pathway, the N-terminal portion of APP is being cleaved by the β-secretase Beta-site APP cleaving enzyme 1 (BACE1), releasing a secreted β-APP portion and the membrane-bound fragment CTF99 (Vassar et al., 1999). Then, the γ-secretase complex, which consists of the four protein subunits presenilin (PSEN1 or 2), presenilin enhancer (PEN), APH, and Nicastrin, cleaves the ε-site of CTF99 generating the Aβ (its dimension depends on the cutting site) peptide and the amino-terminal APP intracellular domain (AICD) (Chow et al., 2010; Long and Holtzman, 2019). In addition, the γ-secretase complex is involved in the cleavage of the remaining Aβ at the C-terminal end, generating a sequence of shorter peptides (e.g., p3 peptide) until Aβ is released from the complex. Mutations in *PSEN1* and *PSEN2* genes have been observed to affect the APP cleavage and thereby result in the alteration of the Aβ production (Wang et al., 2007). The Aβ peptide is mainly produced inside the neurons' endosomes and its release outside the neurons is modulated by synaptic activity (Kamenetz et al., 2003; Wei et al., 2010) at both, pre-synaptic (Cirrito et al., 2008, 2005) and post-synaptic (Verges et al., 2011) levels. Once generated intracellularly, the Aβ peptides are normally distributed in the neuronal cytosol, but also in the organelles involved in the secretory pathways, such as the endoplasmic reticulum (ER), medial Golgi as well as trans-Golgi network (Greenfield et al., 1999; Hartmann et al., 1997). Then, Aβ peptides aggregate into oligomers, protofibrils, fibrils, and finally in senile plaques due to their β-sheet conformation (Fig. 2A). In addition, the Aβ soluble oligomers have been reported to potently inhibit long-term potentiation (LTP), enhance long-term depression (LTD), and reduce dendritic spine density in rodent hippocampal slice cultures (Kamenetz et al., 2003; Wei et al., 2010). The accumulation of Aβ is also involved in the generation of free radicals, such as reactive oxidative species (ROS), that in turn, may damage the protein and lipid fractions within the neuronal membranes together with other intracellular enzymes (e.g., glutamine synthase and creatine kinase) involved in pathways for neuronal function and survival (Yatin et al., 1999).

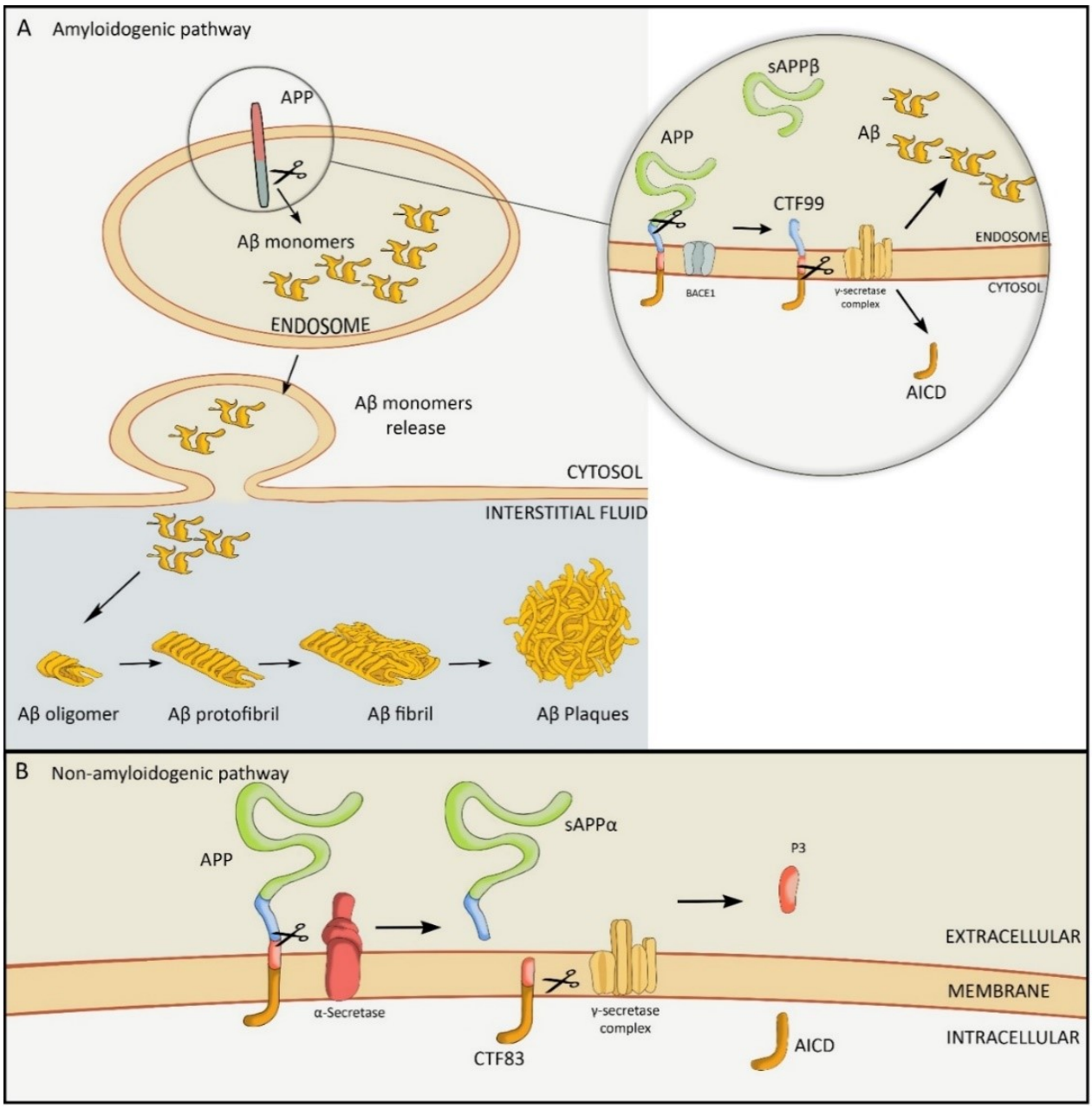



**Figure 2: APP processing in amyloidogenic and non-amyloidogenic pathways.** (A, amyloidogenic pathway) Proteolytic cleavage of APP by BACE1 and γ-secretase is responsible for Aβ monomer production. APP cleavage by BACE1 release sAPPβ in the endosome cytosol. CTF99, the remaining fragment on the membrane, is then cleaved by the γ-secretase complex releasing Aβ monomers in the endosome and AICD inside the neuronal cytosol. Then, Aβ is secreted into the interstitial fluid. High number of Aβ monomers aggregate consecutively into oligomers, protofibrils, fibrils, and plaques. (B, non- amyloidogenic pathway) Proteolytic cleavage of APP by α-secretase releases sAPPα in the endosome or on the cell surface. The remaining CTF83 fragment undergoes subsequent cleavage by the γ-secretase complex, releasing AICD in the neuron cytosol and p3 within the endosome. Abbreviations: Aβ (β-amyloid), APP (amyloid precursor protein), BACE1 (β-secretase Beta-site APP cleaving enzyme 1), sAPPα (soluble N-terminal fragment APP-α), sAPPβ (soluble peptide APPβ), AICD (amyloid precursor protein intracellular domain), CTF83 (carboxyterminal fragments 83), p3 (peptide 3).

#### *1.4.2.3 Neuronal and synaptic loss in Alzheimer's disease*

Synaptic loss is another common feature in the brain of AD patients that parallels the distribution of NFTs and precedes the neuronal loss (Gómez-Isla et al., 1997). Synaptic loss can be visualized by immunohistochemistry with markers for presynaptic proteins (e.g., synaptophysin) and by quantitative electron microscopy (Masliah et al., 1993; Overk and Masliah, 2014; Scheff et al., 2001). Synaptic density has been considered one of the best marker for cognitive decline in AD (DeKosky and Scheff, 1990; Ingelsson et al., 2004; Scheff et al., 2007, 1990; Scheff and Price, 1993; Terry et al., 1994). Indeed, decrease in synaptic density in the hippocampus and medial temporal lobes has been associated with early AD symptoms, such as cognitive and language impairment (Deture and Dickson, 2019). In AD, neuronal transmission may be compromised by synaptic damage and by impairment in the axonal transport due to tau hyperphosphorylation in dendritic spines (Hoover et al., 2010). Interestingly, another study observed that the remaining unaffected synapses become larger and more robust, giving rise to the hypothesis of a compensatory process against AD progression (Serrano-Pozo et al., 2011).
Neuronal loss in the brain areas of entorhinal and prefrontal cortex represents one of the most evident features that correlates with the severity of AD. In particular, a 90% decrease of neuron population was detected in later stages of the pathology (Padurariu et al., 2012; Terry, 2006; Zilkova et al., 2006). Neuronal loss can be visualized in sections either with hematoxylin and eosin or with Nissl stainings and by NeuN immunohistochemistry (Braak et al., 2018; Wolf et al., 1996; Zille et al., 2012).

## 1.5 Mouse models for Alzheimer's disease

Mouse models are strong research tools used for studying neuropathological disorders, such as Alzheimer's and Parkinson's diseases (PD) (Strome and Doudet, 2007; Waerzeggers et al., 2010). As mentioned in paragraph 1.2, the majority of AD cases in humans are sporadic (SAD form) which have a combination of many genetic and environmental factors. Although enormous progress has already been achieved in creating a high variety of mouse models for AD research, most of the widely used animal models are based on the FAD rather than the SAD form (review LaFerla and Green, 2012). However, the biggest divergence between the FAD and SAD form is represented by the age of onset, as the pathological and clinical phenotype resulted indistinguishable in the early onset of the disease (LaFerla and Green, 2012). Several translational issues among animal models and human beings have been reported (LaFerla and Green, 2012). In particular, mouse models are questioned regarding their reliability for studying human diseases due to their different complexity (e.g., different networks that connects genes to disease between animal models and human beings) (Perlman, 2016). The most common translational issues between humans and mouse models are as follows: absence of consistency between preclinical models and human clinical trials; heterogeneity of humans enrolled in clinical trials while the majority of mouse models used are in-bred strains; lack of significant neurons and synaptic loss in the majority of mouse models (the disease prodromal phase is more represented by mouse models); mouse models have a strong genetic background (FAD form), while most of patients develop the SAD form (LaFerla and Green, 2012). To date, the full spectrum of the AD neurological disorder is not completely recapitulated in any single mouse models, while each model allows only in-depth analysis of few elements of the disease (LaFerla and Green, 2012). Despite these limitations, mouse models allow to conduct investigations on AD research when human studies are not readily possible due to ethical reasons (LaFerla and Green, 2012). Transgenic mouse models for AD show similarity with humans in the architecture and function of the hippocampal and entorhinal cortex circuits, that mediate episodic memory, and in the genes' number. This provides a reductionist system capable of facilitating the experimental manipulation (Hall and Roberson, 2012).
Mutations in AD-associated genes (e.g., *APP*, *PSEN1* and *PSEN2*) have been commonly used for most of the AD mouse models (Hall and Roberson, 2012). In particular, mutations in the DNA stretches encoding for two cleavage sites of the *APP* gene are normally involved in the Aβ production. The Swedish mutant (K670N/M671L) is a double mutation at the β-secretase cleavage site encoding region responsible for the increase in the BACE1 cleavage and thus for the increased production of Aβ40 and Aβ42 (Citron et al., 1992; Suzuki et al., 1994). Mutations in the DNA stretches encoding for the γ-secretase cleavage site, responsible for the production of the more toxic Aβ42 in comparison to Aβ40, are represented by the

London's (V717I) and the Indiana's (V717F) ones (Chartier-Harlin et al., 1991; Price and Sisodia, 1998; Suzuki et al., 1994). The Dutch (E693Q) and the Arctic (E693G) mutations increase the fibrillogenesis or the resistance to proteolysis (Massi et al., 2009; Nilsberth et al., 2001). Mutations in genes encoding for *PSEN1* and *PSEN2*, represent one of the main causes of the autosomal dominant Alzheimer's disease (Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995) and of the increase in Aβ42/Aβ40 ratio (Duff, 1997; Oyama et al., 1998; Sherrington et al., 1996, 1995). In addition, the background of the used strains represents another important factor to consider in relation to the mouse model phenotype. Based on endogenous traits of common background strains, such as C57BL/6, 129, FVB/N, DBA, and C3H, there are different levels of anxiety, activity, susceptibility to excitotoxicity, inflammation, neurodegeneration, and learning/memory abilities (Carlson et al., 1997; Gerlai, 1996; Owen et al., 1997; Pugh et al., 2004). Among the mouse lines created, the APPswe/PS1ΔE9 mouse model presents the human *APP* (*hAPP*) gene containing the Swedish mutation and the *PS1* gene containing the ΔE9 mutation, both responsible for amyloid plaques' development and for the consequent AD-like behavioral deficits around 6–7 months of age (Jankowsky et al., 2004). However, a more aggressive mouse model for developing AD in a more rapid time window is the 5XFAD line, which combines five AD-related mutations, including the Swedish (K670N/M671L), the Florida (I716V), and London (V717I) mutations in the *hAPP* gene and the M146L and L286V mutations in the human *PS1* gene (Oakley et al., 2006). In particular, 5XFAD mice show already at the age of 4 months AD pathological hallmarks, such as high levels of Aβ42 and more pronounced cognitive deficits. This is also observed by high number of neuronal loss unlike the APPswe/PS1ΔE9 mice (Oakley et al., 2006).

## 1.6 Current therapeutical approaches for Alzheimer's disease

Despite the enormous numbers of scientific publications on AD research, there are no successful pharmacotherapeutic treatments available. The only available treatments try to counterbalance the neurotransmitter deficits due to the progression of the disease. Currently, the most common and approved treatments for mild to moderate AD forms are acetylcholinesterase inhibitors (AChEIs), such as donepezil (Pfizer, New York, NY, USA), rivastigmine (Novartis, Basel, Switzerland) and galantamine (Janssen, Beerse, Belgium), while for the moderate to severe AD forms, memantine represents the most common treatment (Farlow, 2002; McShane et al., 2019). For the supplementary behavioral symptoms, AD patients have been treated with antipsychotics and antidepressants together with the above-mentioned treatments (Ballard and Corbett, 2010). The AChEIs treatment for AD relies on the assumption that the cholinergic system in the basal forebrain is being affected in the early onset of the disease. In particular, the cholinesterase inhibitors are used to enhance the cholinergic transmission and to delay the degradation of acetylcholine neurotransmitter in the synaptic cleft (Bartus et al., 1982; Cummings and Back, 1998). Improvements in AD cognitive functions have been observed already after the first 3 months of AChEIs treatment together with a less rapid decline in the cognitive functions over the next 3-9 months (Birks, 2006; Hansen et al., 2008). In addition, treatment with AChEIs improves also several symptoms associated to AD, including a reduced attention, memory, praxis, language comprehension and communication (Qaseem et al., 2008). However, it has been observed that the use of AChEIs may induce some side effects in AD patients, such as increased rates of syncope and bradycardia (Gill et al., 2009). Therefore, AChEIs are not considered as a "definitive cure" for AD but just medications that can temporarily reduce the symptoms. The above-mentioned memantine is used as therapeutic treatment for moderate to severe AD and consists of a noncompetitive N-methyl-D-aspartate (NMDA) antagonist drug, which exerts a neuroprotective function against excitotoxicity (Yiannopoulou and Papageorgiou, 2013). In particular, 6 months of memantine treatment induced improvements in cognition, activities of daily living (ADL), and other associated behaviors in AD patients (McShane et al., 2019).
Agitation/aggression and related behaviors associated to AD pathology are commonly treated with antipsychotics (Yiannopoulou and Papageorgiou, 2013). However, the use of antipsychotics in AD therapy has been considered controversial due to higher cerebrovascular morbidity and mortality in patients with dementia (Yiannopoulou and Papageorgiou, 2013). In line with this assumption, the use of antipsychotics increases the risk of hip fracture and pneumonia as well as worsening of cognitive impairment in AD patients (Yiannopoulou and Papageorgiou, 2013).

Novel treatments attempting to block the development of the disease, acting on the Aβ or the tau cascade, are currently under research (review Vaz and Silvestre, 2020). These treatments are based on an active or passive immunotherapy which stimulate the patients' immune system to produce its own antibodies against Aβ or tau (review Vaz and Silvestre, 2020). The main differences between these two therapies consist in frequent re-administrations for passive immunotherapy, while a minimal number of administrations and low antigen dosage is enough to attain a persistent production of Aβ antibodies for active immunotherapy (Winblad et al., 2012). Currently, the active immunotherapy is showing positive results (Vaz and Silvestre, 2020). The most common vaccines available against anti-Aβ are represented by Amilomotide (CAD106), Vanutide cridificar (ACC-001), Lu AF20513, ABvac40 and UB-311 (Vaz and Silvestre, 2020). Safe and satisfactory results in preclinical AD patients were observed for Amilomotide (CAD106), a vaccine composed by multiple copies of a short fragment of Aβ (Aβ1-6) currently in the trial phase II/III (Farlow et al., 2015; Lopez Lopez et al., 2019; Vandenberghe et al., 2017; Winblad et al., 2012). Active immunotherapy for tau, the AADvac1 and ACI-35 vaccines, are currently under investigation ( Vaz and Silvestre, 2020). The passive immunotherapy consists in the injection of monoclonal antibodies directed against Aβ or abnormal forms of tau protein (Vaz and Silvestre, 2020). In the first case, six monoclonal antibodies reached phase III clinical trials, including bapineuzumab, solanezumab, crenezumab, gantenerumab, aducanumab, and BAN2401 (Vaz and Silvestre, 2020). Among these, bapineuzumab and solanezumab were the first monoclonal antibodies to reach the phase III trials (review Vaz and Silvestre, 2020). Recently, aducanumab was approved for medical use in the United States by the Food and Drug Administration (FDA) (Kaplon et al., 2022). Despite the aducanumab approval, controversy opinions regarding its efficiency have been reported and long-term safety studies are required (Walsh et al., 2021). In the passive immunotherapy for tau, four anti-tau antibodies reached the phase II trials, including Gosuranemab, Tilavonemab, Semorinemab and Zagotenemab (Vaz and Silvestre, 2020). However, to date, the use of these antibodies showed promising results in reducing the CSF biomarkers levels and Aβ plaques but without clinical efficacy (Klein et al., 2019; Loureiro et al., 2020; Sevigny et al., 2016; Yang et al., 2019).

## 1.7 The gut microbiota and neurodegenerative diseases

Recently, intestinal health has raised interest in relation to neurodegeneration despite the anatomical distance of this organ system from the brain (Houser and Tansey, 2017; T. Zhang et al., 2018). Different studies suggested that depression, anxiety, autism, as well as neurodegenerative diseases, such as PD and AD, may begin in the gut due to a dysregulation of the gut microbiota (de Lartigue et al., 2011; Grasset et al., 2017; Hu et al., 2016; Sampson et al., 2016; T. Zhang et al., 2018). The gut microbiota is a term describing the combination of a vast number of bacteria, archaea, fungi, and viruses colonizing the human gastrointestinal tract (GIT) that mutually interact with the host in a double beneficial relationship (Bäckhed et al., 2005; Neish, 2009). In particular, more than $10^{14}$ cells (nearly 10 times more than human cells) were estimated for the bacteria residing in the gut (Bäckhed et al., 2005; Gill et al., 2006). The assembly of the gut microbial communities in human beings is a highly complex process orchestrated by different factors in relation to a distinct life period, such as infancy (from birth to 12 months), childhood (3–12 years), puberty/adolescence (12–17 years) and adulthood (18–65 years and > 65 years) (for a review see Valeri and Endres, 2021). The first years of life are mainly characterized by an instable gut microbiota, while increased stability in the composition of gut microbiota is typical of adulthood due continuous adaptation to various environmental factors (Aleman and Valenzano, 2019; Spor et al., 2011). Sexual dimorphism plays an additional role in the development of the gut microbiota and in the maturation of the immune and nervous system throughout the lifespan, influencing the abundance of microbial communities as well as immune and neuro-inflammatory pathways in adult males and females (Jašarević et al., 2016; McCarthy et al., 2017). The gut microbiota offers healthy proprieties to the host, such as the maintenance of the mucosal barrier integrity, protection against pathogens and nutrient supply (e.g., vitamins and fermentation of food) and stimulation of immune reaction (Hou et al., 2022; Thursby and Juge, 2017). Among the functions of the bacteria residing in the GIT, the fermentation of complex carbohydrates, with the consequent generation of short-chain fatty acids (SCFAs), is catalyzed by carbohydrate-active enzymes (Musso et al., 2010). In particular, SCFAs undergo a rapid absorbance by GIT epithelial cells, regulating other host cellular processes, including gene expression, apoptosis,

chemotaxis, proliferation, and differentiation (Corrêa-Oliveira et al., 2016). Among the SCFAs produced by bacteria in the GIT, an important role is played by propionate, acetate and butyrate with an estimated ratio of about 1:3:1, respectively (Louis et al., 2014). Most of the gut anaerobes produce acetate, while different subsets of gut bacteria produce propionate and butyrate throughout different pathways (Louis and Flint, 2017). Propionate is generated by the succinate or propanediol pathway, based on the nature of the sugar, while butyrate is generated by glycolysis and acetoacetyl-CoA starting from carbohydrates (Louis and Flint, 2017). Bacteroidetes mainly produce propionate, while Firmicutes are involved in the production of butyrate (Louis et al., 2014; Macfarlane and Macfarlane, 2003; Morrison and Preston, 2016). In addition, Actinobacteria and Firmicutes are also involved in starch fermentation that may contribute to supplement butyrate production in the colon (Louis et al., 2014). In detail, butyrate is a source for colonocytes energy, and it plays an anti-inflammatory and anticancer role attenuating the bacterial translocation and enhancing the gut barrier function by affecting tight-junction assembly and mucin synthesis (Corrêa-Oliveira et al., 2016; Lin and Zhang, 2017; Morrison and Preston, 2016). Butyrate and propionate act as histone deacetylase inhibitors, regulating epigenetically genes involved in the immune system and in the inflammatory response (Corrêa-Oliveira et al., 2016; Lin and Zhang, 2017; Morrison and Preston, 2016). The only SCFA that is involved in the modulation of the appetite and energy intake is propionate, which attenuates the reward-based eating behavior (Byrne et al., 2016; Chambers et al., 2015). The gut microbiota plays a crucial function in the synthesis of vitamins, which the host is incapable of producing, such as biotin, vitamin K, panthotenic acid, nicotinic acid, riboflavin, pyridoxine and thiamine (Hill, 1997). Among the gut microbes implicated in the synthesis of essential vitamins, lactic acid bacteria exert a vital role in the generation of vitamin B12 (LeBlanc et al., 2013; Martens et al., 2014), while *Bifidobacteria* in the production of folate, a vitamin involved in host metabolic processes, such as the DNA synthesis and repair (Pompei et al., 2007). In particular, *Lactobacilli rhamnosus GG, Lactobacillus plantarum* and *Akkermansia muciniphila* are involved in cell renewal and wound healing as well as promotion of epithelial integrity (Chen et al., 2010; Reunanen et al., 2015; Swanson et al., 2011). Therefore, on one hand, the gut microbiota provides beneficial support to the host, including the improvement of the gut integrity (Natividad and Verdu, 2013), the promotion of metabolic processes (Den Besten et al., 2013), the protection against pathogens (Baümler and Sperandio, 2016) and the regulation of the hosts immunity (Gensollen et al., 2016). On the other hand, the alteration of the microbial composition is known as dysbiosis and it has a negative impact on the host-bacterial interaction mechanisms (Thursby and Juge, 2017). Gut microbiota dysbiosis may be elicited due to exposure to various environmental factors, including diet, toxins, drugs, and pathogens but also via diseases (Carding et al., 2015).

### 1.7.1 The intercommunication between the gut microbiota and the neuronal system

An important factor to consider when talking about the gut microbiota is its interconnection with the brain in the well-known “gut-brain” axis (GBA). This axis fulfills its function via connecting the emotional and cognitive centers of the brain with the intestinal peripheral functions, including the immune activation, intestinal permeability, enteric reflex and the entero-endocrine signaling (Carabotti et al., 2015). The GBA incorporates the central nervous system (CNS), the sympathetic and parasympathetic arms of the autonomic nervous system (ANS), the enteric nervous system (ENS), and the microbiota (Carabotti et al., 2015). Inside this axis, the vagal nerve represents a mediator between the gut microbiota and the brain, influencing the motor, sensory, and secretory pathways but also the visceral messages from the GIT to the brain (Bravo et al., 2011; O’Mahony et al., 2011). This network also includes the interconnection between the hormonal and the neuronal system that affects the activities of intestinal functional effector cells, such as immune cells, epithelial cells, enteric neurons, smooth muscle cells, interstitial cells of Cajal, and enterochromaffin cells (Carabotti et al., 2015). Therefore, the structures and functions of the brain may be modulated by the gut, and contrariwise, the brain may control the gut microenvironment and its microbiota composition (Zhao et al., 2018).

The gut microbiota exerts its impact on the brain, at least in part, via release of some of the major brain neurotransmitters that are involved in the modulation of food intake and energy balance (Silva et al., 2020). Among the neurotransmitters, serotonin controls brain and neuroendocrine functions, such as emotion, cognition, motor function, pain as well as food intake, circadian rhythms and reproductive

activity throughout a modulating mechanism of peristaltic, secretory, vasodilatory, vagal and nociceptive reflexes (Crowell and Wessinger, 2007; Martinowich and Lu, 2008). Gut microbes are also able to influence the brain by producing metabolites, such as SCFAs, that are involved in several functions, including the reinforcement of the blood brain barrier (BBB) integrity and the modulation of neurotransmission (Fung et al., 2017; Silva et al., 2020; Stilling et al., 2016). In particular, SCFAs possess neuroactive properties enabling them to influence levels of neurotrophic factors and to promote memory consolidation (Silva et al., 2020) (Fig. 3A). The GBA also modulates the stress response of the gut in relation to the possible development of gut disorders (Thakur et al., 2014). Moreover, alteration in gastrointestinal functions can lead to visceral events, such as nausea, satiety, and pain due to the gut-brain interaction (Forsythe and Kunze, 2013). As mentioned in the paragraph before, gut microbiota dysbiosis may be the result of several factors described in a variety of disease conditions (Rosenfeld, 2015; Tilg and Moschen, 2014). Gut dysbiosis, induced by pathogen-associated substances in the GIT, may contribute to AD progression by generating inflammatory cytokines (e.g., IL-17) and gut metabolites (e.g., SCFAs) that are associated with increased intestinal barrier and BBB leakage (Seo and Holtzman, 2020). Moreover, such release of cytokines and gut metabolites can induce an amplification of the plasma T helper type 1 (Th1) cells, which may invade the brain tissue and cause an excessive neuroinflammation that can accelerate neurodegeneration and AD pathogenesis (Seo and Holtzman, 2020) (Fig. 3B). Experimental evidence concerning the role of the gut microbiota in AD patients and animal models, together with the underlying molecular pathways, is reported in the next paragraph (chapter 1.7.2). A schematic representation of the potential interplay between the gut microbiota and the brain in physiological and AD conditions is provided in figure 3A and 3B, respectively.

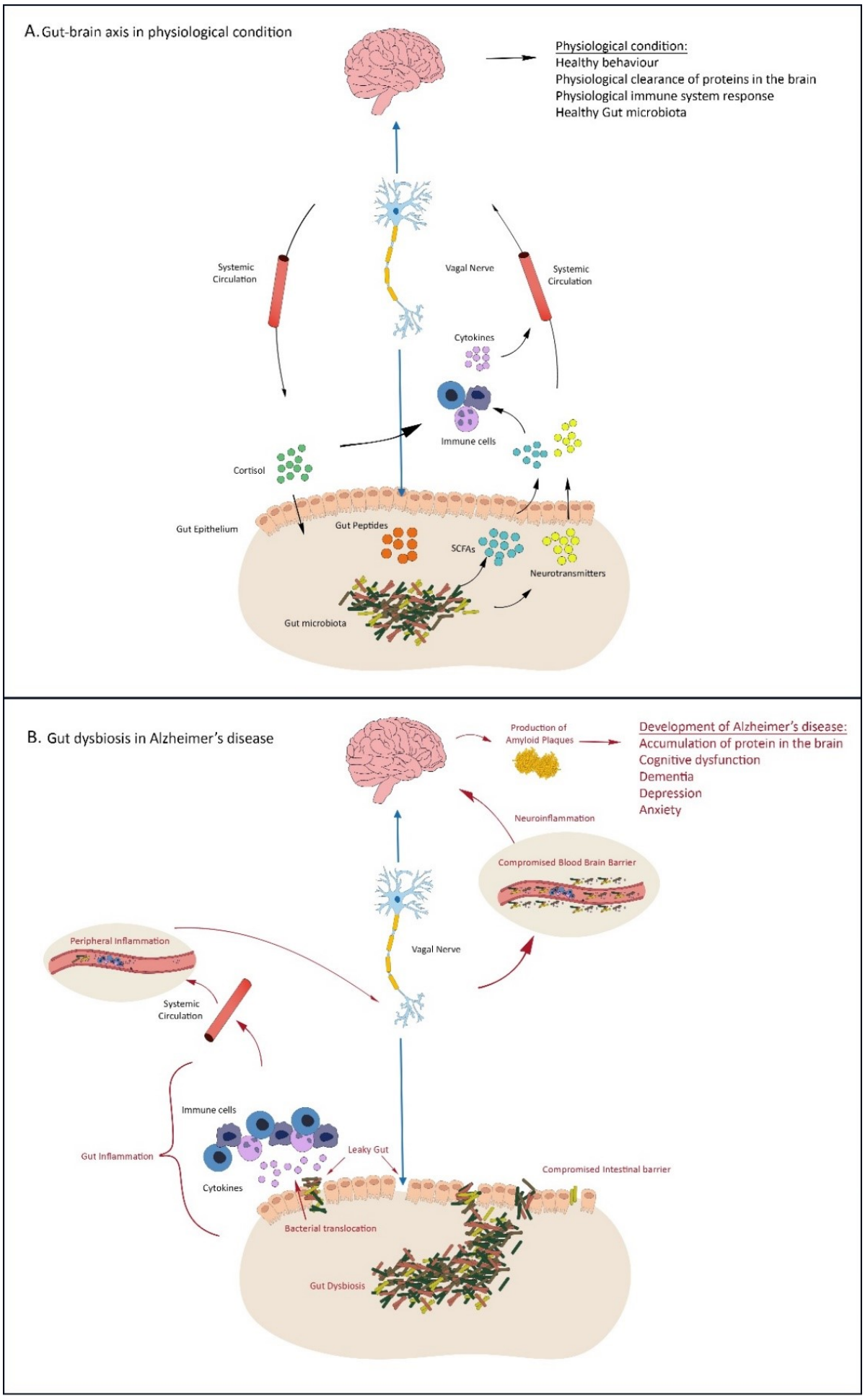

A. Gut-brain axis in physiological condition
Physiological condition:
Healthy behaviour
Physiological clearance of proteins in the brain
Physiological immune system response
Healthy Gut microbiota
Systemic Circulation
Vagal Nerve
Systemic Circulation
Cytokines
Immune cells
Cortisol
Gut Epithelium
Gut Peptides
SCFAs
Neurotransmitters
Gut microbiota
B. Gut dysbiosis in Alzheimer's disease
Production of Amyloid Plaques
Development of Alzheimer's disease:
Accumulation of protein in the brain
Cognitive dysfunction
Dementia
Depression
Anxiety
Neuroinflammation
Compromised Blood Brain Barrier
Peripheral Inflammation
Vagal Nerve
Systemic Circulation
Immune cells
Gut Inflammation
Leaky Gut
Cytokines
Compromised Intestinal barrier
Bacterial translocation
Gut Dysbiosis

**Figure 3: Schematic representation of the GBA functions in physiologic (A) and in AD (B) conditions.** *(*A) In healthy condition, the gut can communicate with the brain through the vagal nerve and the systemic circulation modulating several brain functions, the endocrine signaling (hypothalamus-pituitary–adrenal axis) and the immune response. (B) Gut dysbiosis induces the activation of the immune system together with alteration of neurotransmitter production and bacterial metabolites (e.g., SCFAs and LPS). The gut microbiota increases the GIT permeability ("leaky gut") allowing the translocation of bacteria or bacterial products into the systemic circulation and causing a peripheral inflammation. The inflammation state, once propagated to the brain through the vagal nerve, induces the disruption of the BBB permeability and affects further brain functions. The generated neuroinflammation contributes to AD pathogenesis, increasing the production of senile plaques together with AD symptoms. Abbreviations: SCFAs (short-chain fatty acids).

### 1.7.2 The molecular pathways connecting gut microbiota and Alzheimer's disease

Concerning the relationship between AD pathology and the gut microbiota, several studies have been carried out to assess the molecular pathway mechanisms behind their interconnection. Significant amounts of amyloids, such as the curli proteins, are normally produced by the gut microbiota (Kowalski and Mulak, 2019) sharing similar tertiary structure with the CNS amyloids (Friedland, 2015; Wang et al., 2015). The production of amyloid proteins helps the bacterial cells to bind each other to form biofilms and to resist destruction by physical and immune factors (Cherny et al., 2005; Friedland and Chapman, 2017). Gram negative bacteria residing in the gut release lipopolysaccharides (LPS) and amyloid species from their outer membranes into the local intestinal environment that can polymerize and form insoluble fibrous protein aggregates able to promote oxidative stress (Friedland, 2015; Iadanza et al., 2018; Morales et al., 2013; Oli et al., 2012; Schwartz and Boles, 2013). An example of such mechanism was provided by Asti and Gioglio (2014) which showed that the release of endotoxin from *Escherichia coli* was able to increase the formation of Aβ fibrils in an *in vitro* model. Additionally, they also observed a potentiation in amyloids fibrillogenesis by co-incubating Aβ peptide with LPS (Asti and Gioglio, 2014). Moreover, gut exposition to bacterial amyloid proteins may trigger the immune system response to produce neuronal amyloids in the brain (Friedland and Chapman, 2017). Chen and colleagues showed enhanced neuronal α-synuclein (α-syn) deposition in both gut and brain, and increased microgliosis and astrogliosis in rats exposed to curli-producing *Escherichia coli* compared to controls (rats exposed to bacteria unable to produce curli amyloids) (S. G. Chen et al., 2016). Rats subjected to a chronic LPS injection into the fourth brain ventricle reproduced many of the inflammatory and pathological changes seen in the brain of AD patients (Hauss-Wegrzyniak et al., 2000). In addition, prolonged Aβ production in the hippocampus together with increased cognitive deficits were observed after injection of LPS in the mice peritoneal cavity (Kahn et al., 2012). The presence of LPS has also been reported in the hippocampus and in the neocortex brain regions of AD patients, which colocalizes with Aβ1-40/42 around the blood vessels (Zhao et al., 2017). Interestingly, the LPS concentration in the blood of AD patients was significantly higher than in healthy people, providing another evidence for a possible involvement of the microbiota in AD pathology (Zhang et al., 2019). More in detail, LPS binds the Toll-Like Receptors (TLRs) expressed on microglial cells that in turn interact with CD14 and MD-2 intracellular proteins inducing an inflammatory response (Dua P, 2015). Among the TLRs, the activation of TLR4 is involved in the inflammatory response upon binding Aβ and S100A8/A9 proteins (He et al., 2016), while TRL2 is triggered by Aβ and other bacterial amyloids (Zhao et al., 2017). Recent studies suggested the existence of an overlap mechanism between bacterial amyloids and the human Aβ proteins (Ambrosini et al., 2019). Based on such similarity, bacterial amyloids may act as prion proteins (e.g., curli, Aβ, tau and α-syn) inducing another protein to adopt a pathogenic β-sheet structure. Such mechanism is called "molecular mimicry" and may be the cause of a greater inflammatory response in the AD brain due to altered gut microbiota (Delzenne et al., 2011; Muegge et al., 2011; Rosenfeld, 2015).

As already mentioned, the GBA allows the interaction between the CNS and ENS and with this, the transportation of misfolded proteins in both directions. In detail, the misfolded proteins, once produced in the gut lumen and entered in contact with the intestinal epithelium layer, accumulate in dendritic cells of the Peyer's patches and in other lymphoid follicles (Ano et al., 2009). Then, such misfolded proteins may interact with neurons belonging to ENS and consequently, be finally transported to the brain (Ano et al., 2009). Among the bacteria involved in generating significant amounts of amyloid proteins, *Escherichia coli, Bacillus subtilis, Salmonella enterica, Salmonella typhimurium*, and *Staphylococcus*

*aureus* represent the ones that may contribute to AD pathology through the accumulation of misfolded Aβ oligomers and fibrils (Endres and Schäfer, 2018; Hufnagel et al., 2013; Schwartz and Boles, 2013). Moreover, alteration in the bacteria residing in the gut may affect the production of metabolites (e.g., SCFAs) capable of stimulating the sympathetic nervous system to release serotonin and thus, ultimately influencing the cognitive processes (e.g., learning and memory) in the brain of AD patients (Grider and Piland, 2007). However, to date, it is still unclear whether a disbalance of microbiota commensals and/or the presence of a single pathogen or assembly of different pathogens may affect AD pathology (e.g., *Cytomegalovirus, Herpes simplex virus type 1, Chlamydophila pneumonia, and Helicobacter pylori* pathogens are associated with cognitive decline and AD risk (Bu et al., 2015)).

### 1.7.3 Sex differences in relation to Alzheimer's disease and the gut microbiota

Sex is an important variable in both, human and animal research studies, and one of the major risk factors for AD (see paragraph 1.3). In the last decade, several investigations showed a contributing role of sex differences in shaping the gut microbiome across the human and animal lifespan (for a review see Valeri and Endres, 2021). Sex has been considered more than a simple variable in statistical data analysis and several studies have been started considering sex also as an independent research question for experimental design (Miller et al., 2017). Historically, females were largely excluded from animal experiments due to their different chromosome complement (XX versus XY) and gonadal hormones (ovarian versus testicular secretions) involved (Miller et al., 2017). However, several animal experiments revealed that the gut microbiota influence AD pathology in a sex-specific manner. This suggested that the observed sex differences in AD patients may derive, at least in part, from sex steroids, which exert a protection function from the AD development and symptomology (Mielke et al., 2014; Pinares-Garcia et al., 2018). Female estrogen receptor α (ERα) has been described to co-localize with NFTs in the hippocampus of *post-mortem* AD patients (Wang et al., 2016). In particular, the increased interaction between tau and ERα may inhibit the ERα signaling pathway hindering the neuroprotective effect of estrogens (Wang et al., 2016). The gut may play a crucial role in the ERα signaling pathway, since it secretes β-glucuronidase, an enzyme involved in estrogens de-conjugation, activating estrogens for binding the ERα receptors and thus initiating their downstream pathway (Baker et al., 2017; Flores et al., 2012). Examples of the role of the gut microbiota in relation to sex differences in AD pathology have been explored by Minter et al. (2016) in APPswe/PS1ΔE9 mouse model for AD. Interestingly, decreased number of Aβ plaque in association with the expansion of *Allobaculum, Akkermansia,* and *Lachnospiraceae* bacteria were observed in male APPswe/PS1ΔE9 mice treated with broad spectrum antibiotics compared to females (Minter et al., 2016). In addition, also other AD-related alterations were observed in male mice compared to females, such as increased level of soluble Aβ, altered levels of circulating cytokines and chemokines, reduced plaque-localized glial reactivity and altered microglia morphology (Minter et al., 2016). Dodiya et al. (2019) showed that a perturbation of the gut microbiome due to long-term antibiotics exposition is associated with Aβ reduction and alteration of microglial morphology in male APPswe/PS1ΔE9 mice compared to females (Dodiya et al., 2019). Another study showed significant differences in the gut microbiota composition of transgenic EFAD ($5XFAD^{+/-}$/$APOE^{+/+}$) mice associated to *APOE* genotype and sex (Maldonado Weng et al., 2019). Specifically, the relative amounts of the genera *Prevotella* and *Ruminococcus* were significantly higher in females, while *Sutterella* was higher in males (Maldonado Weng et al., 2019). However, no human studies on the relation between the sex-dependent differences in the gut microbiota composition and AD have been reported yet.

## 1.8 Gut microbiota-based therapies for Alzheimer's disease

The connection between CNS and ENS allows the spreading of diseases in both directions (Chalazonitis and Rao, 2018). PD represents one of the most common examples in which gastrointestinal dysfunctions (e.g., obstipation and delayed gut transit) have been observed in almost 80% of the patients affected (Nair et al., 2018). Therefore, the extensive role of the gut microbiota in brain-related disorders allowed speculations for microbiota-targeted techniques as a potential tool for treatment of different CNS diseases, such as AD and PD (Hazan, 2020; Jing et al., 2021; Long-Smith et al., 2020; Thaiss and Elinav,

2017). Among the gut microbiota-based therapies for AD, the use of psychobiotics (e.g., probiotics, prebiotics, and synbiotics), antibiotics, and fecal material transplant (FMT) represent the most commonly suggested intervention tools (Fig. 4). Probiotics are defined by all the living organisms that, after their administration, confer a health benefit to the host (Hill et al., 2014). Such host health benefit is dependent from the strain and not all probiotics exert beneficial effect on the brain (Kelly et al., 2017; Ng et al., 2018; Östlund-Lagerström et al., 2016). The most common probiotic strains are represented by *Lactobacillus* and *Bifidobacteria*, which have well-defined safety profiles. In particular, some probiotics (e.g., *Lactobacillus acidophilus, Lactobacillus casei*, and *Bifidobacterium bifidum*) ameliorate human mood and depression states (Akkasheh et al., 2016; Benton et al., 2007). The substrate selectively used by microorganisms to confer health benefit to the host is defined as prebiotics (Gibson et al., 2017). Prebiotics mainly consist of fibers, including inulin, fructo-oligosaccharides, galacto-oligosaccharides (GOSs), and resistant starch which are selectively fermented by gut microbes and are not absorbed in the small intestine. Prebiotics intervention has the advantage of potentially being able to improve gut microbial status more globally, as opposed to the use of single- or multistrain probiotics (Long-Smith et al., 2020). The combination of prebiotics and probiotics is represented by the synbiotics (Long-Smith et al., 2020). In particular, the prebiotics within the synbiotics provide a source of fermentable fiber used to improve the viability of the probiotics. The use of synbiotics has been observed to improve functional GI symptoms in a double-blind randomized controlled trial of a PD cohort (Barichella et al., 2016) and to ameliorate some of the gut-related comorbidities related to autism spectrum disorders (ASD) (Sanctuary et al., 2019). Antibiotics are substances able to remove or prevent the bacterial colonization in the human body (Angelucci et al., 2019). In particular, a long-term administration of a broad spectrum of antibiotics can affect the gut microbiota composition reducing its biodiversity and colonization in both, human and animal studies (Angelucci et al., 2019). Human studies showed that antibiotics are frequently associated with neurodegenerative disorder symptoms, such as anxiety, panic attack, depression, psychosis, and delirium (Neufeld et al., 2017). However, the administration of antibiotics is not usually correlated with neuropsychiatric adverse effects (Angelucci et al., 2019). A new therapeutic mode of intervention for AD involves the administration of antibiotics combined with probiotics or FMT. FMT is a common method that uses the transfer of stool or stool-derived bacteria from an organism defined as “healthy” to a recipient diseased subject in order to confer a health benefit directly changing the microbial composition of the latter (Gupta et al., 2016; Kelly et al., 2017; van Nood et al., 2013). This approach is also used in some animal experiments to explore the underlying mechanisms of the neurodegenerative diseases. Experiments concerning the use of pre-/probiotics and FMT in relation to AD are reported in the next two paragraphs.

### 1.8.1 The role of prebiotics and probiotics in Alzheimer’s disease

Recently, an emerging role for treatment of AD and other neurodegenerative disorders has been discussed by prebiotics and probiotics. A clinical study conducted on 60 AD patients reported that probiotics supplemented with *Lactobacilli* and *Bifidobacteria* significantly improved the patients’ Mini-Mental State Examination scores (patients randomly divided into two groups with both $n = 30$ for milk-treated control group and for probiotic group) (Akbari et al., 2016). Among the studies conducted to unveil the effect of the gut microbiota composition on AD pathology, Minter et al. (2016) showed that male transgenic mice subjected to antibiotics treatment, displayed decreased Aβ plaques deposition in the brain and better performance in behavioral tasks for long-term memory assessment compared to control (vehicle). In addition, they confirmed a correlation between changes in the gut microbiota composition, increased microglial activity and AD pathology (Minter et al., 2016). Similarly, exercise training and probiotic supplementation diet in APPswe/PS1ΔE9 transgenic mice improved cognitive performance in Morris Maze test and reduced number of Aβ plaques in the hippocampus (Abraham et al., 2019). Attenuation of learning and memory deficits and reduced Aβ1-42 and tau proteins was also shown in an AD rat model (AD-like symptoms were manifested by treatment with D-galactose (D-gal) and Aβ) administered orally for 4 weeks with a prebiotic fructooligosaccharides from *Morinda officinalis* (OMO) (Chen et al., 2017).

Recently, the effect of a changed microbiome on AD pathological hallmarks upon a period of 14 weeks were investigated in 5XFAD mice treated either with antibiotics or probiotics (*Lactobacillus acidophilus*

and *Lactobacillus rhamnosus*) (Dos Santos Guilherme et al., 2021). Both treatments affected the gut microbiome with antibiotics reducing the number of viable microbes and probiotics transiently increasing Lactobacillaceae. The sole usage of antibiotics improved the quality of nest building (a proxy for assessing hippocampal abilities), reduced amyloid plaque load in the hippocampus, reduced the blood sugar concentration, increased serum glucagon levels together with a reduction in the receptor for advanced glycation end products (RAGE; a mediator of Aβ amyloid transport across BBB into the brain), while the probiotics administration resulted in a null effect (Dos Santos Guilherme et al., 2021).
Finally, the use of a dietary intervention based on antibiotics, prebiotics or probiotics may produce significant effects in the modulation of the gut microbiota that, in turn, may directly affect the ENS and consequently the CNS-related diseases (Perez-Pardo et al., 2017; Szablewski, 2018).

### 1.8.2 Fecal material transplant as a therapeutic option for Alzheimer's disease

FMT is an ancient technique that was described for the first time in the fourth century in China, for treatment of several conditions, such as diarrhea (Zhang et al., 2012). Then, in 1958, it was re-introduced for the treatment of pseudomembranous enemas (Eiseman et al., 1958). While high rate of success is reported for gut disorders, such as *Clostridium difficile* infection (CDI), inflammatory bowel disease (IBD), autoimmune disorders, allergic diseases and obesity (Austin et al., 2014; Choi and Cho, 2016; Kelly et al., 2017; Smits et al., 2013; van Nood et al., 2013) in humans, little is known about the efficacy and the mode of action for the treatment of neurodegenerative disorders. To date, there is not a common universal definition of the "healthy donor" that can be applied for both, bacterial infections and treatment of neurodegenerative disorders. The selection of an healthy donor for fecal microbial transplant to treat bacterial infection due to *Clostridium difficile*, includes exclusion criteria regarding any recent gastrointestinal illness (e.g., colic and diarrhea), medical treatment, or dietary supplementation with probiotics (McKinney et al., 2020). Even more complex is defining an FMT healthy donor for neurodegenerative diseases treatment due to discrepancy in the patients' individual microbiota composition. For instance, it has been reported that AD patients differ in the gut microbiota composition from healthy individuals, with AD patients showing increased Bacteroidetes and decreased Firmicutes and *Bifidobacterium* levels (Vogt et al., 2017). In another study, AD patients showed increased relative abundance of *Ruminococcaceae, Enterococcaceae* and *Lactobacillaceae* and decreased levels of *Lanchnospiraceae*, *Bacteroidaceae*, and *Veillonellaceae* compared to aged-matched healthy patients (Zhuang et al., 2018) . The discrepancy in the gut microbiota composition, observed in these two studies, might be due to differences in methodology (e.g., sample size) and/or parameters, such as lifestyle and dietary habits. Moreover, Haran et al. (2019) reported increased proportion of *Bacteroides, Alistipes, Odoribacter, Barnesiella* and decreased proportion of *Lachnoclostridium* in AD patients, while increased proportions of *Odoribacter* and *Barnesiella* and decreased proportions of *Eubacterium*, *Roseburia*, *Lachnoclostridium*, and *Collinsella* in elders without dementia (Haran et al., 2019). Decreased fecal microbial diversity was reported in AD patients compared to normal healthy controls (Liu et al., 2019). In addition, a significant reduction of Firmicutes and highly enriched levels of Protobacteria phylum were reported in AD patients compared to controls (Liu et al., 2019). As for humans, alteration in the gut microbiota composition was also observed in AD mice models. APPswe/PS1ΔE9 mice revealed an alteration of the microbiota composition and diversity in mice aged between 8 and 12 months. In particular, increased abundance of *Verrucomicrobia*, *Proteobacteria*, *Ruminococcus* and *Butyricicoccus* was observed in APPswe/PS1ΔE9 mice compared to age-matched wild type controls (Zhang et al., 2017). Decreased microbial diversity in APPswe/PS1ΔE9 mice aged between 3 and 8 months compared to wild type controls was also observed (Shen et al., 2017). Interestingly, such reduced microbial diversity was associated to worsening of AD pathology as shown by higher amount of Aβ deposition in the hippocampus and with a performance decline in Morri's water maze spatial test (Shen et al., 2017). Brandscheid and colleagues (2017) revealed an increased Firmicutes/Bacteroidetes ratio in transgenic 5XFAD mice as compared to wild type mice aged 9 weeks. This, together with the presence of Aβ peptide in gut tissue sections of 5XFAD mice, suggests a possible direct influence of Aβ peptides on the gut microbiota composition balance (Brandscheid et al., 2017).
FMT studies conducted on both, humans and animals, presented several limitations due to: the use of different antibiotics for pre-treatment, different FMT procedure, different donor choice and lack of long-

term follow-up or appropriate control groups (Vendrik et al., 2021). The absence of risk factors for infectious or other chronic diseases in healthy volunteers should be considered for an ideal stool donor (Bibbò et al., 2020). Despite the promising results achieved in human gut disorders, the role of FMT as therapeutic option for AD patients is still under debate. To date, no case reports have been published in AD patients except for only a preliminary study (Hazan, 2020). In this case, a 82-year-old man receiving FMT (a single 300 mL infusion of stool from the 85-year-old patient's wife) for recurrent CDI showed improvements in AD symptoms as early as 2 months post-FMT and continued to the 6-month follow-up visit (Hazan, 2020). Similar to AD, only a case report and a preliminary study have been conducted in PD patients (Huang et al., 2019). In detail, the stool obtained from a 26-year-old college student was used to treat a single male PD patient (aged 71 years old) with 7 years of resting tremor, bradykinesia and constipation (Huang et al., 2019). The FMT temporarily (for 1 month) improved leg tremors and other related PD symptoms (e.g., constipation) 1 week after the third FMT administration and reduced the time of defecation (from 30 to 5 minutes) (Huang et al., 2019). However, PD symptoms returned to the base level in the following 3 months post the FMT (Huang et al., 2019). Improvement in motor and non-motor symptoms were also observed in a small study (*n* = 11) conducted by Kuai et al. (2021) on PD patients after FMT (Kuai et al., 2021). In particular, increased level of *Blautia* and *Prevotella* and decreased level of Bacteroidetes were observed in FMT-treated patients over a total period of 12 weeks (Kuai et al., 2021). In addition to the low number of FMT interventions in human patients, a limited number of studies have been conducted in both PD and AD rodent models. A study conducted by Sampson et al. (2016) reported about the involvement of gut microbiota in the regulation of movement disorders due to PD in a α-synuclein-overexpressing (ASO) mouse model. In particular, enhanced physical impairments were observed in ASO mice after administration of fecal microbiota from PD patients compared to ones receiving microbiota transplant from healthy human donors (Sampson et al., 2016). Amelioration of motor functions together with increased neurotransmission and decreased neuroinflammation in the brain striatum were observed in PD mice that received fecal microbiota from healthy mice (Sun et al., 2018). On the contrary, opposite results, such as deterioration of motor functions and decreased striatal neurotransmission, were observed in healthy mice after having received fecal microbiota from PD mice (Sun et al., 2018). Recipient germ-free C57BL/6N mice (aged 4 weeks), transplanted with fecal samples from an AD patient, reproduced the bacterial diversity of the AD donor affecting also the mouse behavior (i.e., reduced performance in the object location test and object recognition test) (Fujii et al., 2019). In addition, attenuation of the learning and memory impairment were also observed in SAMP8 mouse model for AD by modulating the gut microbiota composition with FMT (Zhang et al., 2021). Sun et al. (2019) showed that FMT treatment ameliorated cognitive impairments and reduced Aβ deposition accompanied by increased neuronal plasticity (represented by increased postsynaptic density protein 95 (PSD-95) and synapsin I expression) in the brain of APPswe/PS1ΔE9 mouse model. In detail, such animals were daily administrated with fecal material solution from wild type mice donors for a 4-week period (Sun et al., 2019). Another study showed that ADLP$^{APT}$ mice, orally inoculated with fecal material from wild type mice for 16 weeks, reduced the formation of Aβ plaques, NFTs and glial reactivity in the brain together with improvements in cognitive impairment (Y. He et al., 2020). Overall, these studies showed that FMT can modulate the AD condition in animal models.

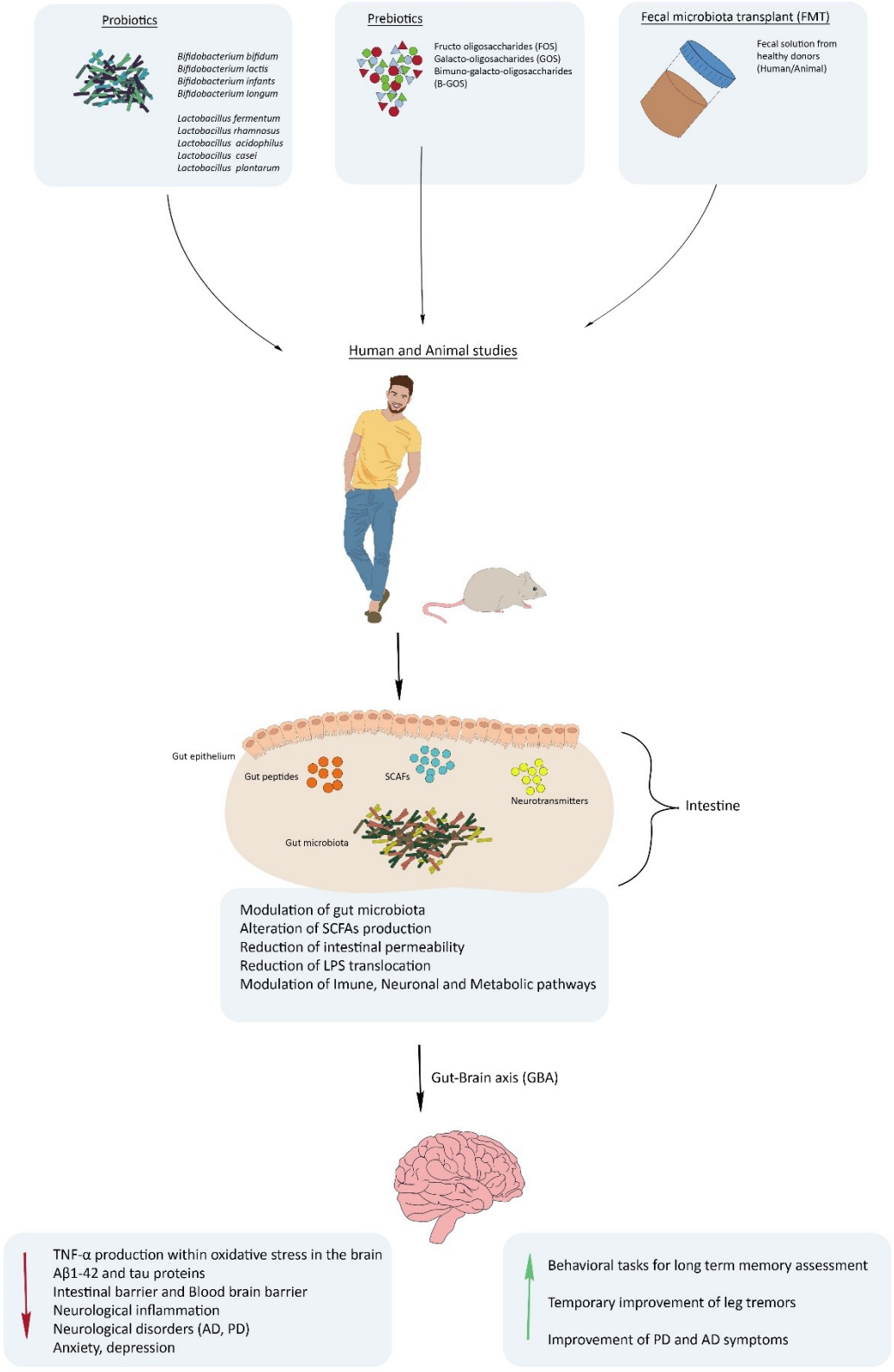



**Figure 4: Scheme of potential gut microbiota-based therapeutical approaches for neurodegenerative disorders.** Human and animal studies showed that administration of probiotics, prebiotics and fecal material from healthy donors modulate gut microbiota composition and might have positive effects on AD and PD patients and on associated symptoms. FMT on AD/PD patients and relative mouse models exert its effects on cognitive deficits and on AD-related hallmarks (e.g., reduction in Aβ deposition in the brain). Abbreviations: SCFAs (short-chain fatty acids), Aβ1-42 (β-amyloid 1-42), AD (Alzheimer's disease), PD (Parkinsons' disease), TNF-α (Tumor necrosis factor α), LPS (lipopolysaccharides).

### 1.8.3 Mouse model studies in gut microbiota research: a focus on translational issues

Mouse models have been used to study the role of the gut microbiota in association with human diseases, including obesity, IBD, autoimmune diseases and neurodegenerative disorders such as, PD and AD (Le Chatelier et al., 2013; Ley et al., 2006; Manichanh et al., 2012; Qin et al., 2003; Vaahtovuo et al., 2008, see chapters 1.7.1 and 1.7.2). Gut microbiota studies have been carried out in humans and mouse models based on their similarity in the physiology and in anatomical structures involved (e.g., organs contained in the gastrointestinal tracts of both species are anatomically similar). Therefore, animal models are considered a useful tool of investigation for addressing the effect of specific microbes on the host (Zhu et al., 2020). However, despite the great progress made in recent years in deciphering the relationship between the gut microbiota and its host, the existence of differences in some key factors between these two systems need to be carefully evaluated when translating results from murine models to humans (Zhu et al., 2020). In particular, humans and animal models present different life experiences, including the exercise regimes, circadian patterns, social pressures and diet (Zhu et al., 2020). While humans diet composition changes daily, mice used as experimental animals are characterized by a controlled and mostly nutritionally monotonous diet (Zhu et al., 2020). In addition, inter-study variations have also been observed in a well-controlled gut microbiota environment using mouse models. Such inter-study variations mostly rely on the mouse house origin, maternal effects, environmental conditions (e.g., diet, light, stress factors and pathogen infection) and genetic backgrounds. Considering the genetic aspects, although humans and mice share more than 85% of their genomic sequences, their gene expression and protein function may diverge (Church et al., 2009; Hugenholtz and de Vos, 2018; Lin et al., 2014; Waterston et al., 2002). Thus, expression of certain genes associated to gut microbiota may also be involved in other external pathways. Such limitations of mouse models do not allow a full recapitulation of human diseases and therefore, they need to be taken into account when translating results from mouse models to humans.

In AD research, genetically modified mice (germ-free, antibiotic-treated, gnotobiotic, and specific pathogen-free) were used to recapitulate some aspects of the human pathology. However, transferability from mouse to human is rather complicated as demonstrated by high percentage of drugs tested in mice that fail in human trials (96.4% from 2002 to 2012; Cummings et al., 2014). Considering the relationship between AD and the gut microbiota, several genes associated to AD were also expressed in the gut (Stoye et al., 2020). Indeed, decrease of acetylcholinesterase activity, normally reduced in brain homogenates (García-Ayllón et al., 2011), was also identified in the small intestine of 5XFAD mice (Stoye et al., 2020). In addition, among 84 gene products associated to AD, lower expression of *apolipoprotein A1* (*ApoA1)* gene and reduced ApoA1 protein content was observed in colon tissue of 5XFAD mice compared to wild type mice (Stoye et al., 2020). It has been suggested the urge of a mouse model with a comparable human gut microbiota and immune system that allows scientists to better translate the findings of gut microbiota-AD interaction into the human system (Schächtle and Rosshart, 2021). Therefore, animal models implicated in translational microbiome research will be required for a more complete description of the microbiome involved (Schächtle and Rosshart, 2021).

## 1.9 The relationship between the gut microbiota and aging

Aging is the predominant risk factor for SAD (see paragraph 1.3) and changes associated with aging may have a potential role in the onset of AD (Jiang et al., 2017). In the adulthood, the gut microbiota is relatively stable but still subjected to everyday lifestyle perturbations (Dethlefsen and Relman, 2011). The gut microbiota composition of adult people over 65 years of age is characterized by increased abundance of Bacteroidetes phyla and *Clostridium cluster IV* compared to younger individuals, while the latter showed higher level of the *Clostridium cluster XIVa* (Claesson et al., 2011). However, the gut microbiota composition changes in the elderly (>60 years old) with a decrease in the level of certain bacteria considered beneficial, including, *Bacteroidetes*, *Lactobacillus*, and *Bifidobacteria* (Hopkins and Macfarlane, 2002; Woodmansey, 2007; Woodmansey et al., 2004). In addition, the gut microbiota of the elderly is characterized by an overall loss of diversity and stability, together with a large degree of variability on the inter-individual species level (Maynard and Weinkove, 2018). The common denominator of such studies is represented by a similarly increased level of *Bacteriodetes* and decreased

Firmicutes phyla with age and by decreased level of *Bifidobacteria* and increased abundance of Proteobacteria compared to younger adults (Biagi et al., 2010; Claesson et al., 2011). Studies on the elderly gut microbiota composition in comparison to younger adults are summarized in Table 2. Aging can also be considered as an immune disorder (Cevenini et al., 2010) in which increased chronic inflammation is induced by low microbial diversity, enrichment in pathobionts and facultative anaerobes, and depletion of Firmicutes (Biagi et al., 2012; Hornef, 2015; Takagi et al., 2019). Overstimulation of both, innate and adaptive immune system, resulted in a low-grade chronic state of inflammation defined "inflammaging" which result in increased gut permeability and in the translocation of bacteria into the systemic circulation (Franceschi, 2007; Franceschi et al., 2000). Moreover, some studies showed that inflammation might be modulated also at the gut epithelium by some specific bacteria, including *Bifidobacterium*, *Lactobacillus,* and *Faecalibacterium* (Bäuerl et al., 2013; Heuvelin et al., 2009; Sokol et al., 2008; Van Baarlen et al., 2011). Such "inflammaging" state may evoke other age-related alterations in the gut microbiota composition, including the gut barrier breakdown and the consequent increase of proinflammatory cytokines and bacteria-derived products in the circulation (Dinan and Cryan, 2017; Köhler et al., 2016). Indeed, high plasma levels of pro-inflammatory cytokines (interleukin IL-6 and IL-8), that are associated to increasement of Proteobacteria and decrease in butyrate producing bacteria were observed in centenarians (Biagi et al., 2012).
Age has also been associated to potential factors inducing gut dysbiosis, such as different type of diet, antibiotic usage, and different living conditions (co-habitation in nursing homes)(Claesson et al., 2011). Gut dysbiosis due to dietary change can induce increased severity of different diseases , such as obesity, colorectal cancer, inflammatory bowel disease, heart failure, type 2 diabetes, and neurodegenerative disorders (Bermúdez-Humarán et al., 2019; Burokas et al., 2015; Kowalski and Mulak, 2019; Mayer et al., 2014). Taken together, aging and gut dysbiosis are both involved in the induction of the inflammation response and are considered as common risk factors for several age-related diseases, including AD (Franceschi, 2007). In addition, the compromising of the BBB, observed during aging, can influence Aβ clearance and neuroinflammation signaling pathways in AD (Elahy et al., 2015; Erdö et al., 2017; Montagne et al., 2015; Oakley and Tharakan, 2014) (Fig. 3B).
In the here presented thesis, Enterobacteriaceae and Lactobacillaceae were used as representative families for investigating the gut microbiota composition in transgenic 5XFAD mice. They can be also seen as counterparts as Enterobacteriaceae comprise pathogenic organisms, while many of the Lactobacillaceae family are acknowledged as probiotics or beneficial commensals (Azad et al., 2018). A more detailed explanation on the role of Enterobacteriaceae and Lactobacillaceae families in relation to aging is reported in paragraph 1.9.1 and 1.9.2.

### 1.9.1 The impact of Enterobacteriaceae on aging

Enterobacteriaceae and other opportunistic facultative aerobes, such as *Staphylococcus* and *Streptococcus*, are the most common human representative bacteria associated to aged-type microbiota (in human beings > 65 years old), while early life stages (infancy and adulthood) are predominantly characterized by high abundance of *Bifidobacterium* (Biagi et al., 2012). The Enterobacteriaceae group include pathobionts, pathogenic bacterial species presented in small concentrations in a physiologic healthy gut, which can prosper in an inflamed gut due to their tolerance to oxygen (Pédron and Sansonetti, 2008). Therefore, because of the aging process, the hosts immune defense may decline in the resistance mechanisms and this allows the Enterobacteriaceae to cause infections (Biagi et al., 2010; Pédron and Sansonetti, 2008). Moreover, it has also been proposed that the increased production of endotoxins in the elderly may be the result of an increased level of Enterobacteriaceae and other gram-negative bacteria that in turn, may induce the inflammatory response (Schiffrin et al., 2010). Among the pathogenic Enterobacteriaceae species, *Klebsiella pneumoniae, Enterobacter cloacae, Staphylococcus aureus,* and *Pseudomonas aeruginosa* have been associated to community-acquired pneumonia in the elderly (Brisse et al., 2009; Remington and Sligl, 2014). Aside the pathogenic species, the Enterobacteriaceae also contain species that are part of the normal flora even though, many of those are mainly associated with diarrhea and other extraintestinal infections (Janda and Abbott, 2000; Leimbach et al., 2013). These infections can be transmitted by fecal-oral or by direct contact with animals and their environment, or by consumption of contaminated food or water (de Graaf et al., 2017). Moreover, several

studies observed that antibiotic treatment, hospitalization, and *Clostridium difficile*-associated diarrhea (CDAD) are responsible for the increase of Enterobacteriaceae in the elderly (> 65 years of age) (Bartosch et al., 2004; Hopkins et al., 2001; Woodmansey et al., 2004).

### 1.9.2 The impact of Lactobacillaceae on aging

*Lactobacilli* are oxygen-tolerant anaerobes consisting in over hundred species described of which 30% harbor the human gastrointestinal (GI) tract (Felis and Dellaglio, 2007). Their functions include the protection of the host against pathogens, the improvement of the intestinal barrier function, and the promotion of healthy metabolic and immunologic pathways in the gut (Walter, 2008). Despite their role in providing benefit to the host, contradictory results have emerged in relation to some *Lactobacillus* species and to the host metabolic homeostasis (Le Roy et al., 2015). For instance, beneficial metabolic functions against obesity and diabetes are provided by *Lactobacillus gasseri* and *Lactobacillus casei,* while other *Lactobacillus* species (e.g., *Lactobacillus reuteri*) have been associated to increased body mass index (BMI) and glycaemia in adults and in host metabolic homeostasis alteration (Kadooka et al., 2010; Matsuzaki et al., 1997; M. Million et al., 2012; ŠtŠepetova et al., 2011). Additionally, some *Lactobacillus* species, belonging to human host with high BMI, have also been characterized for lack of enzymes implicated in the metabolism of carbohydrates (Matthieu Million et al., 2012; Wells et al., 2011).
Interestingly, several studies showed that elders (> 65 years of age) displayed increased viable count of *Lactobacillus* with increased inter-individual variability compared to younger adults (Mäkivuokko et al., 2010; Silvi et al., 2003; ŠtŠepetova et al., 2011; Tiihonen et al., 2010). In particular, higher BMI, increased level of fasting blood glucose and increased total count of *Lactobacilli* were observed in the elderly compared to younger adults (mean age 27 years old) (ŠtŠepetova et al., 2011). In addition, younger adults were more colonized by *Lactobacillus acidophilus* and *Lactobacillus helveticus,* while elderly showed higher prevalence of *Lactobacillus plantarum*, *Lactobacillus paracasei* and *Lactobacillus reuteri* (ŠtŠepetova et al., 2011).
Although *Lactobacilli* seem to play an important role in the elderly gut microbiota composition, to date their genus function on the host metabolism is not clear (Le Roy et al., 2015). Several animal experiments have been carried out to address their functions. Wang et al. (2019) demonstrated that *Lactobacillus paracasei* D3-5 strain, isolated from the infant gut, extends life span of *Caenorhabditis elegans* (fed with $0.3 \times 10^8$ CFU/mL in the culture media) and prevented high fat diet induced metabolic dysfunction, leaky gut, and inflammation in older mice (8–80-week-old) compared to younger ones (6–8-week-old) (Wang et al., 2019). The beneficial effects of *Lactobacilli* have also been observed by He and colleagues (2020), which analyzed the long-term effect of *Lactobacillus casei Zhang (LcZ)* on human adults (aged 18–49; adult-probiotic group) and on human older adults (aged > 50 years) gut microbiota over a period of 12 months. Faster and greater gut microbiota stabilizing effects were observed after *LcZ* ingestion in the adult-probiotic group compared to the older group. In addition, prolonged and continuous *LcZ* exposition shifted the intestinal microbiota age index of the older adults towards the level of the adult-probiotic group suggesting an improvement in the overall gut microbiota composition and structure (Q. He et al., 2020). An animal experiment conducted on male Sprague-Dawley rats showed that, administration of *Lactobacillus plantarum DR7* (induced by administration of D-gal) in a 12-week period ameliorated cognitive and memory functions (e.g., reduced anxiety and enhanced memory during behavioral assessments) in aged rats compared to young rats (Zaydi et al., 2020). Also, a similar study showed that administration of lactic acid bacteria (10 log CFU/rat/day) in aged-induced D-gal rats for 12 weeks, prevented the shortening of telomere length, enhanced lipid, renal, and liver profile in D-gal-treated rats compared to the untreated controls (Hor et al., 2019). Overall, these studies suggested that *Lactobacilli* might be a potential tool for anti-aging-based therapy.

| Age | Elderly | Younger adults | Family/Genus/Species | Phylum | Methods | References |
|---|---|---|---|---|---|---|
| >60 years old | ↑ | 20-50 years old | Enterobacteriaceae | Proteobacteria | 16S rRNA | Mueller et al., 2006 |
| >65 years old | ↑ | 28–46 years old | *Clostridium* cluster IV | Firmicutes | 16S rRNA V4 region | Claesson et al., 2011 |
| | ↓ | | *Clostridium* cluster XIVa | Firmicutes | | |
| | ↓ | 19-35 years old | *Bifidobacterium longum* | Actinobacteria | Quantitative analysis CFU/g | Woodmansey et al., 2004 |
| | ↓ | | *Bifidobacterium catenulatum* | Actinobacteria | | |
| | ↓ | | *Bifidobacterium boum* | Actinobacteria | | |
| | ↓ | | *Bifidobacterium infantis* | Actinobacteria | | |
| | ↑ | | *Bifidobacterium angulatum* | Actinobacteria | | |
| | ↑ | | *Bifidobacterium adolescentis* | Actinobacteria | | |
| >70 years old | ↑ | 25–45 years old | *Lactobacillus reuteri* | Firmicutes | Real-time qPCR | Rahayu et al., 2019 |
| | ↑ | | Enterobacteriaceae | Proteobacteria | | |
| | ↓ | | *Clostridium cocoides* | Firmicutes | | |
| | ↓ | | *Clostridium leptum* | Firmicutes | | |
| | ↓ | | *Bifidobacterium* | Actinobacteria | | |
| | ↓ | | *Prevotella* | Bacteroidetes | | |
| | ↓ | | *Bacteroides fragilis* | Bacteroidetes | | |
| | ↓ | | *Lactobacillus plantarum* | Firmicutes | | |
| 67-79 years old | ↑ | 26–43 years old | | Proteobacteria | 16S rRNA V1–V3 regions | Kim et al. 2019 |
| | ↓ | | | Bacteroidetes | | |
| 60–80 years old | ↑ | 20–40 years old | *Akkermansia muciniphila* | Verrucomicrobia | 16S rRNA | Biagi et al., 2010 |
| 65–83 years old | ↑ | 24–64 years old | *Clostridium* cluster XIVa | Firmicutes | 16S rRNA V3-V4 region | Kong et al., 2016 |
| | ↑ | | Ruminococcaceae | Firmicutes | | |
| | ↑ | | Christensenellaceae | Firmicutes | | |
| | ↑ | | *Akkermansia muciniphila* | Verrucomicrobia | | |
| 69–89 years old | ↑ | 30–46 years old | *Proteus* | Proteobacteria | Quantitative analysis CFU/g | Gavin et al., 2001 |
| | ↑ | | *Providencia* | Proteobacteria | | |
| | ↑ | | *Bifidobacterium adolescentis* | Actinobacteria | | |
| 70-85 years old | ↓ | 21–39 years old | *Rumicococcus* | Firmicutes | 16S rDNA | Mäkivuokko et al. 2010 |
| | ↓ | | *Roseburia* | Firmicutes | | |
| | ↓ | | *Coprabacillus* | Firmicutes | | |
| | ↓ | | *Dialister* | Firmicutes | | |
| | ↑ | | *Lactobacillus* | Firmicutes | | |
| | ↑ | | *Streptococcus* | Firmicutes | | |
| | ↑ | | *Bacteroides* | Bacteroidetes | | |
| 70-90 years old | ↑ | 25-45 years old | *Escherichia coli* | Proteobacteria | Real-time qPCR | Mariat et al., 2009 |
| | ↑ | | *Clostridium leptum* | Firmicutes | | |
| | ↑ | | *Clostridium coccoides* | Firmicutes | | |

**Table 2: Gut microbiota composition of the elderly.** Changes in the relative abundance of elderly microbial taxa in humans were represented with arrows pointing upwards (increase) and downwards (decrease) in comparison to younger adults (< 65 years old). Abbreviations: 16S rRNA (16S ribosomal RNA), Real time qPCR (quantitative PCR).

# 2. Material and methods

## 2.1 Materials

All materials used in the presented thesis are listed in the following sections.

### 2.1.1 Electrical equipment

| **Device** | **Manufacturer** | **Location** |
|---|---|---|
| Analyse scale Quintix 124-1 CEU | Sartorius | Göttingen, Germany |
| ASYS Hitech Expert 96 UV Microplate Reader | Biochrom GmbH | Cambridge, United Kingdom |
| CCD camera | Raytest | Straubenhardt, Germany |
| Centrifuge 5417 R | Eppendorf AG | Hamburg, Germany |
| Centrifuge Megafuge 1.0 R | Heraeus | Hanau, Germany |
| Centrifuge Varifuge 3.OR | Heraeus | Hanau, Germany |
| Chilled steel beads | Qiagen | Hilden, Germany |
| Forma™ STERI-CULT $CO_2$ Incubator | Thermo Scientific | Waltham, USA |
| Halogen floodlight, RITOS type 6095115 AIP44 (150W) | Ritter Leuchten GmbH | Mömbris, Germany |
| Hot Bead Sterilizers | Fine Science Tools | Heidelberg, Germany |
| Infrared thermometer | Braun | Lausanne, Switzerland |
| Liquid nitrogen tank | Thermo Scientific | Waltham, USA |
| Magnetic stirring hotplate MR 3001 K | Heidolph Instruments | Schwabach, Germany |
| Microscope EVOS® XL Core Imaging System | ThermoFisher Scientific | St. Louis, United States of America |
| Microscope: IX50 | Olympus Deutschland GmbH | Hamburg, Germany |
| Microtome Cryostat: 2800E Frigocut | Leica | Walldorf, Germany |
| Mini Trans-Blot® Cell | Bio-Rad | München, Germany |
| Mini-Centrifuge/vortex: Mini-spin MSC-6000 | Biosan | Riga, Latvia |
| Miniplate spinner centrifuge | ThermoFisher Scientific | Waltham, United States of America |
| Motor hand-tool drill for feces homogenization | Xenox | Fähren, Germany |
| Pipette mLine | Sartorius AG | Göttingen, Germany |
| Pipette Research plus | Eppendorf AG | Hamburg, Germany |
| Pipetus | Hirschmann Laborgeräte GmbH & Co. KG | Eberstadt, Germany |
| Pump DC 308 | Anself | Portland, United States of America |
| Real-Time PCR System: StepOnePlus™ | ThermoFisher Scientific | Waltham, United States of America |

### 2.1.1 Electrical equipment

| | | |
|---|---|---|
| Rods | Laborbedarf Bochem | Weilburg, Germany |
| Rotary microtome: Leica RM 2245 | Leica | Walldorf, Germany |
| Stand base | Laborbedarf Bochem | Weilburg, Germany |
| Thermomixer 5436 | Eppendorf AG | Hamburg, Germany |
| Thermomixer comfort | Eppendorf AG | Hamburg, Germany |
| Tissue Lyzer | Qiagen | Hilden, Germany |
| Video camera system | Imaging Source | Bremen, Germany |
| Video camera system | ELP, Shenzen | Guangdong, China |
| Vortex: IKA MS1 shaker | IKA | Staufen im Breisgau, Germany |
| ZOE Fluorescent Cell Imager | Bio-Rad | Feldkirchen, Germany |

### 2.1.2 Dissection and behavioral equipment

| **Equipment** | **Manufacturer** | **Location** |
|---|---|---|
| Accu-Chek | Roche | Basel, Switzerland |
| Anesthesia induction chamber | Somni Scientific | South Park Township, United States of America |
| Braunol | Braun Petzold | Munich, Germany |
| Forceps | Fine Science Tools | Heidelberg, Germany |
| Guillotine DCAP | World Precision Instruments | Sarasota, United States of America |
| ISANA Kids Badewasserfarbe | REWE supermarket | Mainz, Germany |
| Mice food | Ssniff Spezialdiäten GmbH | Soest, Germany |
| Open field arena for Neophobia test | AG Kristina Endres | Department of Psychiatry and Psychotherapy, Johannes-Gutenberg-University Mainz, Germany |
| Paper stripe material for Nesting test | Ssniff Spezialdiäten | Soest, Germany |
| Radial Arm Water Maze apparatus | AG Kristina Endres, slightly modified from *Alamed et al., 2006* | Department of Psychiatry and Psychotherapy, Johannes-Gutenberg-University Mainz, Germany |
| Reusable Feeding Needles | Fine Science Tools | Heidelberg, Germany |
| Scalpel | Fine Science Tools | Heidelberg, Germany |
| Scissors | Fine Science Tools | Heidelberg, Germany |
| Syringes 10 ml | Braun | Lausanne, Switzerland |
| T-maze apparatus | AG Kristina Endres, based on Deacon and Rawlins, 2006 | Department of Psychiatry and Psychotherapy, Johannes-Gutenberg-University Mainz, Germany |

### 2.1.3 Consumable materials

| Material | Manufacturer | Location |
|---|---|---|
| 1.5 ml/ 2 ml reaction tube | Sarstedt AG & Co | Nümbrecht, Germany |
| 5 ml/10 ml/25 ml pipette cellstar | Greiner Bio-One Internation GmbH | Kremsmünster, Austria |
| 96-well plate, transparent | Greiner Bio-One Internation GmbH | Kremsmünster, Austria |
| Adhesive foil | Avantor VWR | Tonopa, United States of America |
| Aluminum foils | REWE supermarket | Mainz, Germany |
| Aquarium air hose, AH 50-400 | TetraTec Instruments GmbH | Steinenbronn, Germany |
| Chilled steel beads | Qiagen | Hilden, Germany |
| Cover glasses | Sigma Aldrich | St. Louis, United States of America |
| EASYstrainer 20 µm | Greiner Bio-One Internation GmbH | Kremsmünster, Austria |
| ECL-Blotting membrane Amersham Hybond ECL | GE Healthcare | Chalfont, Great Britain |
| Falcon tube | Greiner BioOne Internation GmbH | Kremsmünster, Austria |
| Filtered pipette tips (sterile) Premium 10 µl | biozym: SafeSeal | Hessisch Oldendorf, Germany |
| Filtered pipette tips (sterile) Premium 100 µl | biozym: SafeSeal | Hessisch Oldendorf, Germany |
| Nitrocellulose membrane | GE Healthcare | Chicago, United States of America |
| Parafilm | Isolab GmbH | Wertheim, Germany |
| Pasteur pipettes, glass | VWR International | Radnor (PA), United States of America |
| Pipette tips (unsterile) | Eppendorf AG | Hamburg, Germany |
| Pipette tips (unsterile) | Sarstedt AG & Co. | Nürmbrecht, Germany |
| Rotilabo ® cotton swab | Carl Roth GmbH & Co. KG | Karlsruhe, Germany |
| Selective plates for Enterobacteriaceae and Lactobacillaceae | 3M Deutschland GmbH | Heidelberg, Germany |
| Slide Mounting Media | Thermo Scientific | Waltham, United States of America |
| Super Frosted Microscope Slides | Sigma Aldrich | St. Louis, United States of America |
| SuperFrost Plus® slides | Thermo Fisher Scientific | St. Louis, United States of America |
| Syringes 1ml Injekt-F | Braun | Lausanne, Switzerland |
| TC-dish 100 | Sarstedt AG & Co. | Nürmbrecht, Germany |
| Tupperware | REWE supermarket | Mainz, Germany |

### 2.1.4 Substances

| Substance | Manufacturer | Location |
|---|---|---|
| 10x Taq-Extra-buffer | VWR International S.r.l. | Milano, Italy |
| 4', 6-diamidino-2-phenylindole (DAPI) | Sigma Aldrich | St. Louis, United States of America |
| Acrylamide Rotiphorese® Gel 30 | Carl Roth GmbH & Co. KG | Karlsruhe, Germany |
| Albumin Fraktion V (BSA) | Carl Roth GmbH & Co. KG | Karlsruhe, Germany |
| Ampicillin | Cayman Europe | Tallinn, Estonia |
| Antigen Retrieval Buffer 1 | Medac | Wedel, Germany |
| Anti-Mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor 488 Conjugate) | Abcam | Cambridge, United Kingdom |
| Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) | Fluka Chemie AG | Buchs, Germany |
| Cefaperazone | Cayman Europe | Tallinn, Estonia |
| Colistin | Sigma Aldrich | Steinheim, Germany |
| Deoxynucleotide (dNTP) Solution Mix | NEB Biolabs | Ipswich, United States of America |
| D-Glucose | Merck KGaA | Darmstadt, Germany |
| D-Glucose | Sigma Aldrich | St. Louis, United States of America |
| Disodium phosphate ($Na_2HPO_4$) | Carl Roth GmbH & Co. KG | Karlsruhe, Germany |
| DNase/RNase-Free Distilled Water | UltraPure™, Thermo Fisher Scientific | Waltham, United States of America |
| ECM gel from Engelbreth-Holm-Swarm murine sarcoma | Sigma Aldrich | St. Louis, United States of America |
| Entellan | Merck KGaA | Darmstadt, Germany |
| Eosin Y | Sigma Aldrich | St. Louis, United States of America |
| Ethanol | Carl Roth GmbH & Co. KG | Karlsruhe, Germany |
| Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) | Sigma Aldrich | St. Louis, United States of America |
| Eva Green (50X) | Jena Bioscience | Jena, Germany |
| Fetal bovine serum (FCS) | Gibco by Life Technologies | Carlsbad, United States of America |
| Gentamicin | Sigma Aldrich | Steinheim, Germany |
| Glial cell-derived neurotrophic factor (GDNF) | OriGene Technologies GmbH | Herford, Germany |
| Glycerol | Merck KGaA | Darmstadt, Germany |
| Hemalum solution acid acc. to Mayer (Hematoxylin solution) | Carl Roth GmbH & Co. KG | Karlsruhe, Germany |
| HRP-labeled secondary anti-mouse | Thermo Scientific | Waltham, USA |
| HRP-labeled secondary anti-rabbit | Thermo Scientific | Waltham, USA |
| Hydrogen chloride (HCl) | Merck KGaA | Darmstadt, Germany |
| Isoflurane (Forene Isofluran) | AbbVie | North Chicago, United States of America |
| LBP ELISA assay | Origene | Rockville, United States of America |

### 2.1.4 Substances

| | | |
|---|---|---|
| Magnesium chloride Heptahydrate ($MgCl_2 \cdot 7H_2O$) | Carl Roth GmbH & Co. KG | Karlsruhe, Germany |
| Magnesium sulfate heptahydrate | Carl Roth GmbH & Co. KG | Karlsruhe, Germany |
| Methanol | Honeywell Riedel-de Haën | Seelze, Germany |
| Metronidazole | Sigma Aldrich | Steinheim, Germany |
| $MgCl_2$ (magnesium chloride) (25 mM) | Thermo Fisher Scientific | Waltham, United States of America |
| Milk buffer | Carl Roth GmbH & Co. KG | Karlsruhe, Germany |
| Mouse anti-NOS1 antibody | Santa Cruz Biotechnology | Dallas, United States of America |
| Mouse anti-β-Amyloid antibody 6E10 | Covance | Princeton, United States of America |
| Neomycin | Cayman Europe | Tallinn, Estonia |
| NuPAGE LSD buffer (1X) | Life Technologies | Carlsbad, United States of America |
| Paraformaldehyde (PFA) 4% | AG C. Braun | FZI Johannes Gutenberg-University Mainz, Germany |
| PBS1X (Phosphate Buffered Saline) for mice oral gavage | Gibco, Thermo Fisher Scientific | Waltham, United States of America |
| Potassium chloride (KCl) | Carl Roth GmbH & Co. KG | Karlsruhe, Germany |
| Potassium dihydrogenphosphate ($KH_2PO_4$) | Merck KGaA | Darmstadt, Germany |
| Primer 5XFAD_for/5XFAD_rev (10 µM) | AG Kristina Endres | Department of Psychiatry and Psychotherapy, Johannes-Gutenberg-University Mainz, Germany |
| Protease inhibitor | Roche | Basel, Switzerland |
| Rabbit anti-BDNF | Alomone | Jerusalem, Israel |
| Rabbit anti-GAPDH antibody | Cell Signaling Technology | Danvers, United States of America |
| Rabbit anti-GFAP antibody | Cell Signaling Technology | Danvers, United States of America |
| RNAlater | QIAGEN | Hilden, Germany |
| Roti-Nanoquant | Carl Roth GmbH & Co. KG | Karlsruhe, Germany |
| Sodium bicarbonate ($NaHCO_3$) | Merck KGaA | Darmstadt, Germany |
| Sodium chloride (NaCl) | Carl Roth GmbH & Co. KG | Karlsruhe, Germany |
| Sodium chloride solution (NaCl 0.9%) | B. Braun Melsungen AG | Melsungen, Germany |
| Sodium Citrate ($Na_3C_6H_5O_7$) | Sigma Aldrich | St. Louis, United States of America |
| Sodium hydroxide (NaOH) | Merck KGaA | Darmstadt, Germany |
| Sodium phosphate monobasic dihydrate ($NaH_2PO_4 \cdot 2H_2O$) | Merck KGaA | Darmstadt, Germany |
| SuperSignal West Femto chemiluminescent substrate | Thermo Fisher Scientific | Waltham, United States of America |
| Tamoxifen | Cayman Chemical Company | Ann Arbor, United States of America |

### 2.1.4 Substances

| | | |
|---|---|---|
| Taq DNA polymerase glycerol free | VWR International S.r.l. | Milano, Italy |
| Thioflavin-T (ThT) | Sigma Aldrich | Steinheim, Germany |
| Tissue-Tek® OCT Compound | Sakura Finetek | Torrance, United States of America |
| Tris-HCl, pH 8.0 | Thermo Fisher Scientific | Waltham, United States of America |
| Triton X100 | Merck KGaA | Darmstadt, Germany |
| Tween 20 | AppliChem GmbH | Darmstadt, Germany |
| Vancomycin | Cayman Europe | Tallinn, Estonia |
| Xylene | Carl Roth GmbH & Co. KG | Karlsruhe, Germany |

### 2.1.5 Solutions

| **Solution** | **Composition** |
|---|---|
| Antibiotics' solution (ABXs): high dosage (High ABXs) | 8.3 mg Gentamicin (0.083 mg/mL)<br>4.2 mg Vancomycin (0.042 mg/mL)<br>16,7 mg Metronidazole (0.167 mg/mL)<br>4.2 mg Neomycin (0.042 mg/mL)<br>8.3 mg Ampicillin (0.083 mg/mL)<br>50000 U Colistin (500 U/mL),<br>8.3 mg Cefaperazone (0.083 mg/mL)<br>In 100 ml drinking tap $H_2O$ |
| Antibiotics' solution (ABXs): intermediate solution for pregnant dams (2/3 of High ABXs) | 33.5 ml of High ABXs<br>In 16,5 ml of drinking tap $H_2O$ |
| Antibiotics' solution (ABXs): low dosage (Low ABXs) | 1 ml of High ABXs<br>49 ml of drinking tap $H_2O$ (1/50 of High ABXs) |
| DAPI (4', 6-diamidino-2-phenylindole) | 5 mg DAPI<br>500 µl distilled $H_2O$ |
| Eosin 0,5% | 0.5 g Eosin Y<br>in Ethanol 95% |
| Ethanol 30% | 30 ml Ethanol<br>70 ml distilled $H_2O$ |
| Ethanol 70% | 70 ml Ethanol<br>30 ml distilled $H_2O$ |
| Ethanol 80% | 80 ml Ethanol<br>20 ml distilled $H_2O$ |
| Ethanol 95% | 95 ml Ethanol<br>5 ml distilled $H_2O$ |
| Ethanol 96% | 96 ml Ethanol<br>4 ml distilled $H_2O$ |
| Glycerin (glycerol/PBS1X 10%) | 1 ml Glycerol/PBS1X 50%<br>9 ml argon-flushed PBS1X (1:10 Glycerol/PBS1X 50%) |
| Glycerol/PBS1X 50% | 5 ml Glycerol<br>5 ml argon-flushed PBS1X |

### 2.1.5 Solutions

| | |
|---|---|
| Krebs solution | 6.9 g Sodium Chloride (NaCl)<br>0.343 g Potassium Chloride (KCl)<br>0.203 g Sodium phosphate monobasic dihydrate ($NaH_2PO_4 \cdot 2H_2O$)<br>0.296 g Magnesium chloride Heptahydrate ($MgCl_2 \cdot 7H_2O$)<br>2.1 g Sodium bicarbonate ($NaHCO_3$)<br>0.368 g Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)<br>1.98 g D-Glucose<br>In 1L sterile autoclaved $H_2O$ |
| PBS (Phosphate Buffered Saline) | 5 g NaCl<br>0.2 g KCl<br>1.44 g $Na_2HPO_4$<br>0.24 g $KH_2PO_4$<br>0.133 g $CaCl_2 \cdot 2H_2O$<br>0.10 g $MgCl_2 \cdot 6H_2O$<br>in 1L sterile autoclaved $H_2O$ |
| PBS-Triton X100 0.3% | 300 µl Triton X100<br>in 1L PBS (Phosphate Buffered Saline) |
| PCR lysis buffer (pH 12) | 25 mM Sodium hydroxide (NaOH)<br>0.2 mM Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) |
| PCR Mix | Mix for one sample (22.5 µl):<br>2.5 µl 10x Taq-Extra-Buffer<br>0.75 µl $MgCl_2$ (25 mM)<br>0.5 µl dNTPs<br>0.25 µl Primer mix (10 µM for Rev and For)<br>2.5 µl Lysate tissue<br>0.125 µl Taq-Polymerase<br>0.5 µl EvaGreen (50x)<br>17.875 µl PCR- $H_2O$ |
| PCR neutralization buffer (pH 5) | 40 mM Tris-HCl |
| Thioflavin-T (ThT) 1% | 1 g Thioflavin-T (ThT)<br>in 100 ml of Ethanol 80% |
| Tris buffered saline (TBS) 10X (pH 7.5) | 100mM Tris HCl<br>1.5M NaCl<br>diluted in 1L of autoclaved $H_2O$ |

### 2.1.6 Software

| **Software** | **Manufacturer** | **Location** |
|---|---|---|
| Aida image analyzer 4.26 software | Raytest | Straubenhardt, Germany |
| Anymaze (version 6.1) | Stoelting Europe | Dublin, Ireland |
| ImageJ<br>(Web page: https://imagej.nih.gov/ij/) | Rasband, W.S | Maryland, United States of America |
| Microsoft Excel | Microsoft | Washington, United States of America |
| Prism 6 and 8 | Graph Pad Inc. | San Diego, United States of America |

### 2.1.7 Animals

| **Animals** | **Manufacturer** | **Location** |
|---|---|---|
| ARC-creERT2/+.R26CAG-LSL-Sun1-sfGFP-Myc/+<br>(Termed as Arc-sfGFP)<br><br>Published in:<br>Guenthner et al., 2013 | received from AG J. Winter | Institute of Human Genetics, University Medical Center (UMC), Mainz |
| B6SJL-Tg (APPSwFlLon, PSEN1*M146L*L286V), termed as "5XFAD"<br><br>Published in:<br>Oakley et al., 2006 | Purchased from Jackson Lab (Bar Harbor, United States of America)<br><br>own breeding: AG Kristina Endres | Department of Psychiatryand Psychotherapy, Johannes-Gutenberg-University Mainz, Germany |

## 2.2 Methods

All methods presented in the thesis were published in short form in the following original works: Valeri et al. (2021); Nguyen et al. (2021), and Dos Santos Guilherme et al. (2022). A detailed description of methods directly used for this thesis is provided in the following sections.

### 2.2.1 Experimental animals

Animals used in the presented thesis comprise transgenic 5XFAD mice and respective wild type littermates (published in Valeri et al., 2021; Nguyen et al., 2021) and Arc-GFP-Sun-Cre mice (published in Dos Santos Guilherme et al., 2022). A detailed description of the strains is provided in the following sections.

#### *2.2.1.1 Transgenic 5XFAD mice*

The transgenic 5XFAD mouse model (code: B6SJL-Tg (APPSwFlLon, PSEN1*M146L*L286V)6799Vas/Mmjax)) for investigating on familial AD was purchased from Jackson laboratory (Bar Harbor, United States of America). A detailed description of mutations and translational issues related to 5XFAD mice was already provided in the introduction section (see chapter 1.5). The procedure regarding the breeding and maintenance of 5XFAD mice was already described in Reinhardt et al. (2018). In brief, maintenance of the strain was obtained by crossbreeding with female wild type C57B/6J mice (animal facility of the Department of Psychiatry and Psychotherapy, Johannes-Gutenberg University of Mainz, Germany), followed by crossing wild type females with heterozygous transgenic males. All animals were genotyped by qPCR analysis of DNA extracted from the ear biopsies collected with animal labelling. Mice of both sexes were used in all the described following experiments. Moreover, the male and female littermates that resulted as negative for the FAD mutations (see chapter 1.5) were used as wild type controls in behavioral tests.
All animal procedures regarding the care and the use of animals for experimental procedures were approved by local authorities (Landesuntersuchungsamt Rheinland-Pfalz; approval number G 17-1-035) and carried out in accordance with the European Communities Council Directive guidelines (§4 of the German animal welfare act). For mouse maintenance, controlled standard conditions were applied. This included the presence of *ad libitum* availability of solid food (Ssniff Spezialdiäten GmbH, Soest, Germany) and water, routine of 12 h of light/dark cycle room, temperature (21–23 °C), humidity and air exchange. The number of animals entering the single experimental procedures was calculated taking into account the outcome of former behavioral experiments (e.g., pharmacological interventions).

#### *2.2.1.2 Transgenic Arc-GFP-Sun-Cre mice*

Transgenic Arc-GFP-Sun-Cre (termed as “Arc-sfGFP”) mice (code: ARC-creERT2/+.R26CAG-LSL-Sun1-sfGFP-Myc/+; described in Dos Santos Guilherme et al., 2022) were obtained from the animal house of the Johannes Gutenberg University (TARC) by crossbreeding of Arc-GFP and Sun-Cre mice (purchased from Jackson laboratory (Bar Harbor, United States of America) via the working group of Jennifer Winter, Human Genetics, UMC Mainz). Administration of Tamoxifen (Cayman Chemical Company, Ann Arbor, United States of America) within these mice activated CRE-mediated recombination, leading to expression of a GFP-tagged isoform of the immediate early gene Arc in the nucleus (Guenthner et al., 2013).
Mice of both sexes were used in the described following experiments. Mice were held on a 12-h light/dark cycle and group-housed with a maximum of four mice per cage, with *ad libitum* access to food (Ssniff Spezialdiäten GmbH, Soest, Germany) and water. All animal procedures regarding the care and the use of animals for experimental procedures were approved by local authorities (Landesuntersuchungsamt Rheinland-Pfalz; approval number G 17-1-035 or G 17-1-021) and carried out in accordance with the European Communities Council Directive guidelines. The animals were kept in the animal house of the TARC (JGU Mainz).

### 2.2.2 Antibiotic treatment regime

Transgenic 5XFAD mice were subjected to antibiotics treatment in order to reduce the amount of bacteria residing in the gut. The entire procedure is graphically represented in figure 7A. One week after mating, male 5XFAD mice were removed from the breeding cage in order to provide more space for the pups and to start the antibiotic treatment in pregnant dams. After two weeks from mating, pregnant mothers were administered a mixture of antibiotics until the third week of pregnancy. The antibiotics treatment regime was adapted from (Minter et al., 2017) excluding Kanamycin due to its teratogenic properties (Holdiness, 1987) and changing the mode of administration (from daily oral gavage to *ad libitum* administration in the drinking bottle) to avoid potential undesired side effects (e.g., stress due to daily mice handling and fixation) as described elsewhere (C. S. Wu et al., 2021). The mixture of antibiotics (high ABXs) included reagents at their following final concentrations in tap water: Gentamicin (0.083 mg/ml), Vancomycin (0.042 mg/ml), Metronidazole (0.167 mg/ml), Neomycin (0.042 mg/ml), Ampicillin (0.083 mg/ml), Colistin (500 U/ml), and Cefaperazone (0.083 mg/ml) (Sigma Aldrich, Steinheim, Germany or Cayman Europe, Tallinn, Estonia). In the last week before birth, antibiotics dosage was furtherly reduced to 2/3 of the initially described dosage and adapted to the mean daily drinking volume of a mouse (4 ml) in order to mitigate possible health and safety risks caused by a prolonged administration. At the pups' birth, antibiotics mixture was further reduced to 1/50 of the initial dosage (low ABXs). Autoclaved food and low ABXs were administered as *ad libitum* until mice weaning. Dams and mice pups' health and welfare were daily monitored along the entire duration of the antibiotics' treatment (until mice weaning, P21) according to procedures described elsewhere (Burkholder et al., 2012).

### 2.2.3 Caecum content preparation

Wild type littermates of the 5XFAD strain at the two time points of 1 month (termed as "young") and 12 months (termed as "old") of age were designated as donors for the caecum microbiota transplant. Caecum content preparation procedure is published in Valeri et al. (2021). A description of the procedure is provided in the following sections. In detail, mice were anesthetized by isoflurane (Forene Isofluran, AbbVie, North Chicago, United States of America) inhalation and sacrificed by cervical dislocation. The caecum preparation procedure was adapted from Ellekilde et al. (2014). Mice were dissected placing the mouse in the dorsal recumbent position and making an incision in the midline region (Fig. 5). The caecum region of the intestine was isolated, and the caecum content freshly collected in pre-weighed Eppendorf tubes (stored on ice for the entire procedure) already filled with glycerin (glycerol/PBS1x 10%) solution. Prior to caecum content collection, glycerin solution was constantly flushed with argon gas for 10 minutes in order to remove the presence of dissolved oxygen and to allow colonization of different groups of bacteria, such as Lactobacillaceae and Enterobacteriaceae, under anaerobic conditions (da Cunha et al., 2019; Vasiliadou et al., 2020). The resulting caecum suspension was divided into aliquots (100 µl stock) and shock-frozen in liquid nitrogen. Thereafter, aliquots were stored at −80°C for a maximum of 1 month for preserving the intact properties of the microbiota composition as described elsewhere (Gweon and Na, 2021). Mice donors of both sexes (one male and one female) were used for the preparation of the caecum content to balance the effect of sex on the gut microbiota properties in the recipient mice. Caecum content transfer was termed as "FMT" based on previous investigations (Gacias et al., 2016; Kim et al., 2020; N. Li et al., 2020). Aliquots were further diluted 1:5 with fresh Argon-flushed PBS prior to oral gavage and 150 µl of the resulting solution were administered per mouse to 5XFAD mice.

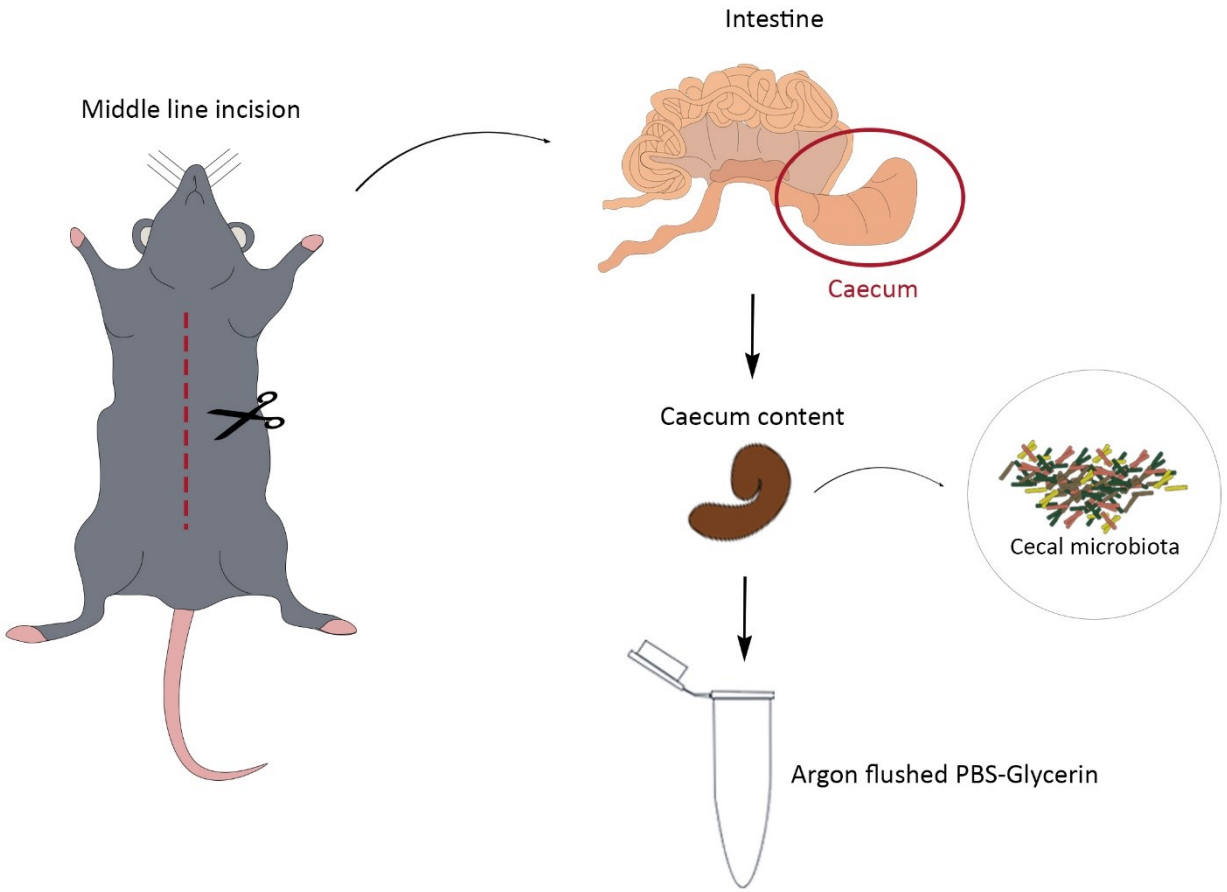



**Figure 5: Caecum content preparation procedure.** Mice were anesthetized by isoflurane and placed in the dorsal recumbent position. A middle line incision was performed on the mouse skin and the caecum content of the intestine collected in pre-weighed Eppendorf with glycerin solution.

### 2.2.4 Cecal microbiota transplant (FMT) procedure

FMT procedure is represented in figure 7A. At three weeks of age (P21), mice pups were weaned, genotyped, and divided by sex in cages containing a maximum of five animals. Then, 5XFAD mice were further divided in single cages and used for the experimental procedure (starting from P22). To avoid experimental bias, 5XFAD mice of both sexes (50% of males and females) were selected and maintained on ABXs regime and on autoclaved food for one week (until P28). At four weeks of age (P28), ABXs treatment was substituted by autoclaved tap water until the next day (P29) to remove residues of antibiotics treatment and to prepare the mice intestine for the imminent colonization with the host cecal microbiota. On the following day (P29), transgenic male and female 5XFAD mice were randomized according to the future assigned treatment: control (no antibiotics treatment; mock treatment: PBS/Glycerol), young (caecum content from 1-month-old wild type littermates), old (caecum content from 12-month-old wild type littermates). The randomization in groups was performed taking into account the order of the ear numbers and the mice sex to avoid any experimental bias. Mice were acclimatized in a quiet animal room for 15 minutes (avoiding any stressful noise) before proceeding with FMT administration. FMT was performed by single acute oral gavage administration in the morning (around 10:00 AM). Age-matched wild type mice received a single mock-gavage (PBS/Glycerol solution) treatment to mimic the FMT procedure and therefore, to simulate the same stressful experience as the inoculated transgenic mice. Transgenic 5XFAD mice that received a mock treatment were also used for a direct comparison of viable bacterial representatives with the young and old

FMT-treated 5XFAD mice. Mice showing any sign of health impact (e.g., ragged fur, low body weight) were excluded from the FMT experiment. All animals were single-caged directly after the FMT or mock-administration (P29) in new fresh cages to prevent transfer of gut microbiota by coprophagy.

### 2.2.5 Quantification of bacterial CFUs in fecal samples

Mice fecal samples were collected and the Lactobacillaceae and Enterobacteriaceae families were evaluated by counting colony forming units (CFUs) on selective plates (3M Deutschland GmbH, Heidelberg, Germany) following the manufacturer instructions. Quantification of bacterial CFUs was performed as described in Dos Santos Guilherme et al. (2020). Briefly, fresh fecal pellets were collected from mice in the afternoon at 3:00 pm in ice-contact tubes and homogenized with a drill hand-held device (Xenox, Fähren, Germany) in a 0.9% sodium chloride solution (100 µl per mg feces). Bacterial suspension was further diluted with isotonic sodium chloride solution in a different way for Lactobacillaceae and Enterobacteriaceae. For Lactobacillaceae two consecutive dilutions were applied to reach a final concentration of 0.001 mg/ml (dilution factor 1000). For Enterobacteriaceae a single dilution was applied to reach a final concentration of 0.27 mg/ml (dilution factor 3.667). 1 ml of the final bacterial suspension was spread on selective plates and incubated overnight at 37 °C. Lactobacillaceae and Enterobacteriaceae CFUs were counted after 20 hours from the incubation start time. Bacterial growth was monitored at different time points: one day before, 1 week and 6 weeks after cecal transplant (Fig. 7A). Fecal samples were also investigated in antibiotics-treated and untreated dams, 5XFAD mice that did not undergo pre- and postnatal antibiotics treatment and wild type donors. Colonies were counted from whole plates (Enterobacteriaceae) or representative sectors of plates (Lactobacillaceae) and normalized to fecal material weight.

### 2.2.6 Bacteria analysis by quantitative Real Time-PCR (qPCR)

A detailed microbiological analysis of the groups of bacteria residing in fecal samples from FMT-treated 5XFAD mice and wild type mice donors was conducted at the MVZ Institut fuer Mikrooekologie GmbH (Herborn, Germany). Microbial DNA extraction and quantitative Real Time-PCR (qPCR) analysis were carried out as described elsewhere (Brandscheid et al., 2017; Valeri et al., 2021).

### 2.2.7 Behavioral tests

Behavioral tests are an essential tool for assessing cognitive deficits associated with AD in patients and rodent disease models (Webster et al., 2014). These deficits affect learning, memory, daily living activity, and related cognitive behaviors such as anxiety and exploration. Moreover, worsening of cognitive behavior has also been associated to aging in AD mouse models (Konsolaki et al., 2016). Among the cognitive tasks that have been modeled to assess reference memory, working memory, and executive function after FMT treatment in mice Nesting test, T-maze, Neophobia test, and Radial arm water maze have been used. The battery of behavioral tasks was performed with FMT-treated 5XFAD and sham-treated wild type mice at a final age of 10 weeks and within the last 1.5 weeks before sacrifice following a fixed time schedule (e.g., T-maze always in the morning time) as reported in figure 7B. To avoid experimental bias, cages did not include information regarding the treatment. All mice were randomly organized in groups alternating their sex, genotype (transgenic or wild type) and treatment (cecal content from young or old wild type donors) received.

#### *5.2.7.1 Nesting test*

Nest building is a task used to assess general animal wellbeing, including the assessment of pain (Jirkof, 2014) but also, to evaluate cognitive integrity (Deacon, 2006; Lin et al., 2007). Indeed, Nest building rely on nest-like structure in the hippocampus as reported by several studies, in which hippocampal lesions as well as hippocampal defects and scrapie infection have been associated to worsening of constructions and deterioration of nest quality in mice (Chen et al., 2005; Jedynak et al., 2012; Kondratiuk et al., 2013).
The introduction of material in the home cage allows the evaluation of the quality of the nest building ability (Tappe-Theodor et al., 2019). In addition, this task allows to evaluate the effect obtained within the active, dark phase (used for building the nest) directly in the morning during the inactive, light phase (Tappe-Theodor et al., 2019). Since the Nesting test can be easily customized by removing and re-introducing new nesting material and repeated daily (Tappe-Theodor et al., 2019), a scheme describing the procedure during the entire week is provided in figure 7B. In brief, on the first day (Saturday, around 5:00 PM), mice were individually introduced in a new fresh cage with a paper towel and 10 g of paper stripe materials (Ssniff Spezialdiäten, Soest, Germany). Paper towels and paper stripe materials were always located in the same position. On the following day (Sunday, around 5:00 PM), both the paper stripes and the paper towels were removed, and 10 g of new nesting material was introduced again and kept in the cages for the following two days (Monday and Tuesday). On Wednesday, mice were kept in empty cages overnight (no nesting material and paper towels). Since the nest exert an important role in the pup's thermoregulation (Lapp et al., 2020), the described absence of nesting material and paper towels has been used as a motivation factor for build construction before the evaluation day. The day before the evaluation (Thursday, around 5:00 PM), 10 g of new paper stripe material was introduced in the cages. On the scoring day (Friday morning around 9:00 AM), the quality of the nest building was rated (from 1 to 5; 1 for lower and 5 for highest value; see Deacon, 2006; Hess et al., 2008) and the non-integrated material weighed. Nest evaluation was performed blindly with two observers involved. As mice nest building ability also depends on body temperature, this was measured soon after the nest evaluation (around 9:00 AM) by an infrared thermometer (Braun, Lausanne, Switzerland) in the anal-genital region.

#### *5.2.7.2 T-maze*

The T-maze consists of an enclosed T-shaped apparatus used to assess the cognitive ability of rodents (Deacon and Rawlins, 2006). Once placed at the base of the T, mice are left to explore and eventually choose one of the two goal arms. Alternation in choosing one and then the other of the two goal arms in two consecutive trials is called "spontaneous alternation". This alternation normally occurs in mice without any brain lesions (especially in the hippocampus), reflecting memory of the first choice (Deacon and Rawlins, 2006).
The T-maze procedure was adapted from Deacon and Rawlins (2006). The task was performed always in the morning starting at 9:30 AM for three consecutive days (from Monday to Wednesday; Fig. 7B). On the first day, mice were habituated for 3 minutes in the start arm without access to the goal arms (a removable guillotine door was placed at the end of the start alley). Mice were tested in the next two days using the same procedure. Briefly, the same mouse was tested with two consecutive trials given in quick succession. A total of 2 minutes for each trial was given to each mouse to choose one of the two goal arms. In the first trial, a removable central partition was placed directly in the middle of the two goal arms (and extending right into the start arm) to produce higher alternation rates in mice (Deacon et al., 2003). Once the mouse reached the chosen goal arm, a removable guillotine door was placed behind it to close the alley and to forbid it from going back to the start arm. Afterwards, mice were placed in their home cages. In the second trial, the central separation door was removed, and the goal arm choice of the mice was tested again. After that, mice were placed in their original home cages. Between tests, the T-maze apparatus was cleaned with a 30% ethanol solution to avoid any experimental bias (e.g., odors) affecting the choice of the next tested animal. Additional support to detect the entry in the different arms was provided by a video camera system (ELP, Shenzen, Guangdong, China) coupled with the Anymaze software (version 6.1; Stoelting Europe, Dublin, Ireland).

#### *5.2.7.3 Neophobia test*

The emergence Neophobia test is used to investigate anxiety and exploratory behaviors in rodents (Paré et al., 2001). It is considered an adaptation of the open-field test which is based on the rodent's innate tendency to exploration as well as aversion to brightly lit open spaces (Paré et al., 2001). The task involves the observation of the time required for mice to exit a dark and closed space and to explore the surrounding enlightened arena (Paré et al., 2001). Normally, mice with high anxiety levels tend to spend more time inside the container (safe space) rather than in the open space (Paré et al., 2001).
More in detail, a black acrylic box (10x10 cm) with an aperture of 5 cm length and 5 cm height was placed in an empty open field arena (60 cm length, height and width) with a white surface. The arena was enlightened for the entire duration of the test with a single strong spotlight (halogen floodlight, RITOS type 6095115 AIP44, 150W, Ritter Leuchten GmbH, Mömbris, Germany). Mice were positioned in the black acrylic box with the main entrance closed with a removable door. After 2 minutes, the exit door was removed and the time the mouse needed to be outside of the box with all four paws was measured (in seconds). Between tests, the box and the arena were cleaned with ethanol solution (30%) to avoid any experimental bias.

#### *5.2.7.4 Radial arm water maze (RAWM)*

The radial arm water maze (RAWM) is a behavioral task used in rodents to assess rapid learning without requiring electrical shock-induced fear conditioning or food deprivation as motivating factors. Despite the existence of many tasks used to address memory impairment in transgenic AD mice, the RAWM is considered as the most reliable task to distinguish different grades of learning (i.e., discrimination between strong and weak learning in mice) (Alamed et al., 2006). Unlike the canonical radial arm maze, RAWM motivates mice to quickly reach the location of the platform by immersion into water. This allows a fast assessment of learning deficits associated to errors in the localization of the goal arm and to delay to reach the platform (Alamed et al., 2006).
Mice were tested in the early afternoon (around 1:30 pm) as described in figure 7B. The RAWM procedure was adapted from Alamed et al. (2006) performing the entire protocol in one day instead of two and by reducing the total number of trials from 15 to 7. For the RAWM, a circular pool (97 cm diameter and 40 cm height) with six swim arms (35 cm long each) radiating out from an open central area (30 cm diameter) was provided. Water was made opaque using a non-toxic red bath tablet (ISANA Kids Badewasserfarbe, Mainz, Germany) and the temperature kept constant at 20 °C. The choice of red color for the bath tablet was taken considering the less visual sensitivity of mice to red spectrum (around 600 nm wavelength)(Peirson et al., 2018). This allowed a better masking of the platform used in the test. Two escape platforms, a hidden (submerged below the water level) and a visible (emerged above the water level) one, were alternated in the target goal arm until the fifth trial, while in the last two trials (the sixth and the seventh one) only the hidden platform was used. The development of a spatial strategy for finding a way out from the RAWM is required when mice are tested with the hidden platform. Moreover, further orientation support for the mice was provided by visual cues placed on the walls surrounding the pool. A limit of 60 seconds was given to each mouse to find the platform in the goal arm. Whenever the mice did not reach the platform at the appointed time, they were gently conducted by hand to the platform. Once they reached the platform, mice were kept on it for 10 more seconds. Then, mice were recovered in empty cages with free access to a paper towel and red warming lights to dry them out. Additional support to detect the mice entry in the different arms as well as the swimming time and speed was provided by a video camera system (ELP, Shenzen, Guangdong, China) coupled with the Anymaze software (version 6.1; Stoelting Europe, Dublin, Ireland). Mice speed was monitored throughout the duration of the test to exclude effects of the antibiotic treatment on locomotor activity (average speed: 0.09998 ± 0.03656 m/s for wild type; 0.09793 ± 0.01585 for young FMT-treated group; 0.1045 ± 0.02838 for old FMT-treated group; no significant differences were detected between groups, $p > 0.83$).

### 2.2.8 Tissue collection

Wild type and transgenic mice around 10 weeks of age (at P71 and six weeks after FMT treatment), were anesthetized by a short incubation in a chamber filled with isoflurane (Forene Isofluran, AbbVie, North Chicago, United States of America) vapor inhalation and sacrificed by guillotine decapitation (World Precision Instruments, Sarasota, United States of America). Truncal blood was rapidly collected, and blood sugar concentration (mg/dl) measured from a droplet by a blood glucose guide meter (Accu-Chek, Roche, Basel, Switzerland). The residual blood was allowed to clot by leaving it at room temperature for a minimum of 45 minutes. The clot was removed by a first centrifuge at 1680 g and 10°C for 10 minutes. Then, the obtained serum was centrifuged again at 15680 g and 10°C for 10 minutes and the resulting serum collected and stored at -80°C.
The intestine duodenum and the abdominal fat were collected by performing a middle line incision on the skin of mice, already placed in the dorsal recumbent position, while the brains were fastly collected by opening the skull. Abdominal fat was collected in empty pre-weighed tubes and weighed after being cleaned with PBS1X. A portion of duodenum and one brain hemisphere were firstly cleaned with PBS1X and fixed in 4% paraformaldehyde (PFA) for 24 hours for immunostaining. Afterwards, PFA was removed, and duodenum samples and brain hemispheres were transferred into a 30% sucrose/PBS solution for 48 hours for immunofluorescence staining or embedded in paraffin at 58 °C for immunohistochemistry staining. The other brain hemisphere and the intestinal fecal samples were rapidly collected in RNA later (QIAGEN, Hilden, Germany) and finally stored at -80°C to be used for further analyses.

### 2.2.9 Lipopolysaccharide-binding protein (LBP) ELISA

LBP ELISA analysis was carried out in serum samples from FMT-treated 5XFAD mice by [redacted] (Department of Psychiatry and Psychotherapy, Johannes-Gutenberg-University Mainz) following the manufacture instruction (Origene, Rockville, Maryland, USA). The procedure behind was described in Valeri et al. (2021).

### 2.2.10 Immunostaining analysis of intestine and brain samples

The duodenum region of the small intestine and the brain hemisphere samples were investigated for anatomic morphological analysis and immunostaining for AD hallmarks, respectively. The relative procedures are reported in the following sections.

#### *2.2.10.1 Hematoxylin and eosin staining and analysis*

Duodenal samples were freshly collected at the day of sacrificed and kept in 4% PFA for 24 hours. Paraffin embedding preparation and Hematoxylin and eosin staining of intestine samples were performed by [redacted] (Core facility Immunohistochemistry of the University Medical Center Mainz) and the procedure behind was described in Valeri et al. (2021) and Nguyen et al. (2021). Pictures were captured at 4X and 20X magnification using EVOS XL microscope (Life Technologies, Darmstadt, Germany). Morphological analysis of investigated duodenum anatomical structures (i.e., villus length, crypt depth, muscular layer, and submucosa thickness) was carried out in FMT-treated mice and in mock-treated (received PBS1X solution by oral gavage to mimic the same stress of FMT-treated mice) transgenic 5XFAD and wild type mice. Four regions were selected and analyzed in every slice keeping the same spatial coordinates and following a clockwise orientation to avoid selection bias (structures were measured at 3, 6, 9, and 12 h when seeing the gut cross-section as a clock). The described measurements were conducted blindly, and the related statistical analysis was performed by Graph Pad Prism 6 (San Diego, United States of America).

#### *2.2.10.2 Aβ plaques staining in brain slices*

Brain samples were subjected to paraffin and optimal cutting temperature (OCT) embedding for immunohistochemistry and immunofluorescence staining, respectively. Staining procedures were reported in the following subchapters.

##### *2.2.10.2.1 Paraffin-embedding and immunohistochemistry staining procedure*

Paraffin embedding preparation and immunohistochemistry of brains with 6E10 antibody (Covance, Princeton, United States of America) were performed by [redacted] (Core facility Immunohistochemistry of the University Medical Center Mainz) and the procedure behind was described in Valeri et al. (2021). Two pictures per mouse were captured at 4X and 10X magnification using EVOS XL microscope (Life Technologies, Darmstadt, Germany). Brain target regions (i.e., dentate gyrus, subiculum, cortex and pre-frontal cortex) were examined for densitometric analysis after experiment blinding using Aida image analyzer 4.26 software (Raytest, Straubenhardt, Germany). Unspecific background was subtracted for every picture.

##### *2.2.10.2.2 OCT embedding and immunofluorescence staining procedure*

After PFA fixation, brain hemispheres were directly transferred into 30% sucrose solution and stored at 4°C for at least 48 hours (until they sank at the bottom of the tube). Then, sucrose solution was removed, and brain hemispheres were wiped, frozen in liquid nitrogen and stored at -80°C. Brains were placed inside the cryostat (2800E Frigocut, Leica, Walldorf, Germany), embedded with Tissue-Tek® OCT Compound (Sakura Finetek, Torrance, United States of America) and left there to slowly reach the temperature of -20°C. After OCT solidified, brains were cut in 40 µm sagittal sections and mounted on SuperFrost Plus® slides (Thermo Fisher Scientific, St. Louis, United States of America).
Thioflavin-T (ThT) staining was performed following Ly et al. (2011). In brief, brain sections were allowed to completely air-dry prior proceeding with ThT staining. A series of ascending graded concentration of ethanol (from 70 to 80%) was used to wash each section for 1 minute. Then, each slide was incubated with 100 µl of prefiltered (filter of 0.2 µm) 1% ThT solution for 15 minutes in a dark chamber at room temperature. Sections were washed with a series of descending graded concentrations of ethanol (from 80 to 70%) and with distilled water (1 minute for each step). Glass slides were mounted with Entellan (Merck Chemicals GmbH, Darmstadt, Germany) and stored in a dark slide holder at 4 °C. Brain images were acquired using Zoe Fluorescent Cell Imager (Biorad, Feldkirchen, Germany) microscope. ThT intensity was analyzed by ImageJ software (Rasband, W.S., ImageJ, United States of America) subtracting an established unspecific background (ImageJ rolling ball radius: 50 pixel) to every picture and adjusting for the same threshold values of brightness and contrast.

### 2.2.11 Protein analysis: extraction, Bradford assay, and Western blot

For the biochemical analyses, several proteins involved in AD pathology were investigated on Western blot. Protein extractions, Bradford assay and Western Blot analysis of prefrontal cortex brain regions in FMT-treated mice were performed by [redacted] (Department of Psychiatry and Psychotherapy, Johannes-Gutenberg-University Mainz). The procedure behind was described in Valeri et al. (2021).

### 2.2.12 Longitudinal muscle (LMMP) isolation and analysis of enteric neurons activity in Arc-sfGFP mice

Arc-sfGFP mice were administrated Tamoxifen (Cayman Chemical Company, Ann Arbor, United States of America) by intraperitoneal injection (150 µl, final concentration of 10 mg/ml) in the morning (around 5:30 am) to activate the Arc-GFP expression through CRE-mediated recombination. This step was carried out 5

(for males) and 9 (for females) hours before the social interaction test as also described elsewhere (Dos Santos Guilherme et al., 2022). Mice were euthanized by a short incubation in a chamber filled with isoflurane (Forene Isofluran, AbbVie, North Chicago, United States of America) vapor inhalation and sacrificed by guillotine decapitation. A middle line incision was performed in the abdominal region (see Fig. 5). Longitudinal muscle (LMMP) belonging to the ileum region of mice small intestine was separated from circular muscle in a freshly prepared Kreb solution (see chapter 5.1.5) and placed on a glass slide for subsequent analysis of neuronal activity by fluorescent staining signal. Four images expressing sfGFP positive neurons within *Myenteric plexus* (attached to the LMMP) were visualized by green-fluorescent light and acquired from ileum sections (four sections for each mouse) using the Zoe Fluorescent Cell Imager (Bio-Rad, Feldkirchen, Germany). A total of three defined areas (square measuring 888 pixels for width and height) were designed in each section and number of sfGFP positive neurons analyzed by ImageJ software (Rasband, W.S., USA). An established unspecific background (ImageJ rolling ball radius: 50 pixel) staining was subtracted to every image (Fig. 6).

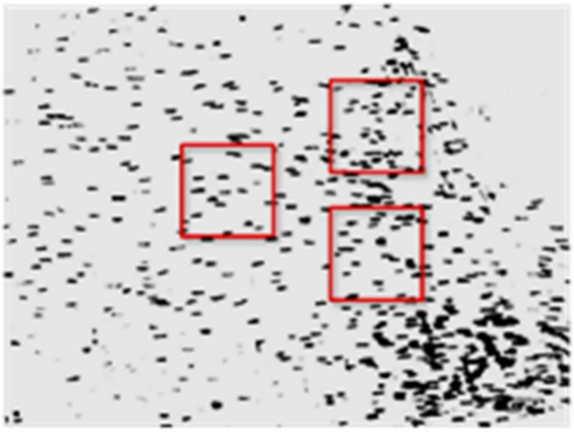


**Figure 6: Method of analysis of Arc-sfGFP positive neurons in ileum myenteric plexus.** Neuron expressing Arc promoter activity via sGFP expression in the myenteric plexus of mice subjected to chronic social defeat stress (CSDS) and not subjected to CSDS (control) were analyzed from a defined area (888 x 888 pixel, three areas indicated in red) for each animal by ImageJ (Rasband, W. S., United States of America)**.** Method published in Dos Santos Guilherme et al., 2022.

### 2.2.13 Statistical analyses

Microsoft Excel (Microsoft, Washington, United States of America) and Graph Pad Prism versions 6 and 8 (Graph Pad Prism Inc., San Diego, United States of America) were used for data collection and statistical analysis, respectively. Data were collected and edited by Microsoft Excel, while statistical analysis and graph generation were performed by using Graph Pad Prism. Statistical significance of the obtained result was assessed either by Student's t-test or One Way ANOVA depending on the presence of multiple comparisons. Additional Welsh-correction and Fisher's LSD post-hoc tests were applied if needed. Data are always expressed as mean ± SEM. Normalization (in % of young group) was applied to ELISA LBP, body temperature, brain staining, and Western blot analyses setting the young group to 100%. Anderson–Darling or Shapiro–Wilk tests were used to assess whether data belongs to a specific or a normal distribution, respectively. A normal distribution of data and presence of outliers (ROUT method with a Q of 1%) were determined by Graph Pad Prism. Levels of statistical significance (p-value) were attributed to values < 0.05 as indicated in Table 3. Mice were assigned to different FMT treatment groups (cecal content from "young" or "old" wild type mice and control wild type mice) alternatingly to ascending ear marking numbers and sex prior to genotyping.

| Significance level | p-value |
|---|---|
| ns, not significant | $p > 0.05$ |
| * | $p < 0.05$ |
| ** | $p < 0.01$ |
| *** | $p < 0.001$ |

**Table 3: Level of statistical significance.** The division of the p-value for different significance levels.

# 3. Aim of the thesis

In the last decade, the intercommunication between the gut and brain became a new research avenue for neurodegenerative diseases, including AD. FMT represents a promising and intriguing tool in AD and related neurodegenerative disorders (see chapter 1.8). In addition, aging has always been considered the biggest risk factor in AD sporadic form and an intriguing question to address also in relation to the gut microbiota. During aging, the bacteria residing in the gut undergo changes in their composition and diversity that may ultimately affect the brain of AD patients. The aim of the here presented thesis was to investigate whether the transplant of microbiota belonging to healthy aged mice may affect the resident microflora and consequently the phenotype of young recipient transgenic 5XFAD mice. Firstly, the composition of the gut microbiota should be evaluated between healthy wild type (C57BL/6) at two time points of 1 month (mice designated as "young") and 12 months (mice designated as "old") of age. In particular, C57BL/6 mice aged 12 months and above have been considered comparable to middle-aged humans between 38 and 55 years old (Dutta and Sengupta, 2016; Flurkey et al., 2007). In addition, they start to exhibit impairments in spatial learning and memory functions comparable to what happen in human adults aged between 60-70 years old (Barrash, 1994; Shoji et al., 2016). Hence, a parallel analysis on donors' and recipient fecal microbiota should be carried by quantitative PCR (qPCR), to recognize whole bacteria phyla or singles species involved, and by counting the Colony forming units (CFUs) of the chosen representative bacteria on selective plates, to rapidly monitor the effect of FMT overtime. Enterobacteriaceae and Lactobacillaceae should be used as representative families as they exert an opposite role on the host health (see chapter 1.9). Recipient 5XFAD mice should be chronically pre-treated with a cocktail of antibiotics to severely reduce the amount of the pre-existing bacteria residing in the gut and to allow the colonization of the donors' microbiota (procedure following Minter et al., 2016). Once assessed the efficiency of the antibiotics' treatment, 5XFAD mice should undergo FMT and the effects of the donors' microbiota on the host should be monitored over a defined period. In this case, a period of 6 weeks should be set as a threshold for assessing the engraftment of the donors' microbiota in the recipient mice as previously reported (see Ellekilde et al., 2014; Le Roy et al., 2019). An intriguing question regards the possibility that a microbiome belonging to an aged host may aggravate the symptoms in mice with AD background. Transgenic 5XFAD mice exhibit memory decline (e.g., learning and memory deficits) and anxiety- and depressive-like behaviors that are associated to AD pathology (Locci et al., 2021). Therefore, a battery of behavioral tasks should be planned to assess the effect of FMT on cognitive functions using wild type mice, not subjected to FMT treatment, as control. Aging is often accompanied by alteration in body temperature with high variability in humans (Balmain et al., 2018; Waalen and Buxbaum, 2011). Among the behavioral tasks that depends, at least partially on the body temperature, the nesting test represents an indicator of mice health and well-being but also hippocampal integrity (Gaskill et al., 2013). Moreover, alteration of certain bacteria in elderly (see Table 2) is associated with gut dysbiosis and impaired intestinal epithelial integrity, which may be responsible for increased gut permeability ("leaky gut") and systemic endotoxemia (Ragonnaud and Biragyn, 2021). Thus, *post-mortem* assessment of the bacterial cell wall component (i.e., the endotoxin LPS) and intestinal morphology parameters (e.g., villi length, crypt depth, submucosa and muscularis thickness) should be addressed in FMT-treated mice. Whether FMT from old donors may induce aggravation of AD hallmarks in the brain of recipient 5XFAD mice is still an open question. Finally, histological examination and quantitative analysis of AD-related brain areas (e.g., cortex, prefrontal cortex, hippocampal dentate gyrus, and subiculum) and different hallmarks involved both in AD pathology and neuroinflammation should be investigated.

Among the factors contributing to the acceleration of AD pathogenesis, exposure to stress has been associated to increased APP expression and generation of Aβ peptides in rodents (review by Justice, 2018). Stress response is connected to the hypothalamus-pituitary-adrenal (HPA) system that in turn, influences the microbiome. It may be plausible that stressful events perceived by the CNS may reach the intestine via the vagal nerve or soluble mediators. Moreover, changes in fecal microbiota composition were observed in C57BL6/J mice subjected to chronic social stress (Bharwani et al., 2016). Therefore, the effect of chronic social stress paradigm (described in Dos Santos Guilherme et al., 2022) should be assessed counting the number of activated neurons residing in *myenteric plexus* layers within the longitudinal muscle (LMMP) of both male and female Arc-sfGFP mice.

# 4. Results and discussion

The mechanisms behind the sporadic form of AD are enigmatic as several factors are contributing to the development of the pathology (see chapter 1.2 and 1.3). Among these factors, the gut microbiota represents an intriguing new field to investigate due to its intercommunication with the CNS in the well-known described GBA. FMT represents a poorly explored therapeutic tool for the treatment of neurodegenerative diseases, including PD and AD, that is based on the gut microbiota manipulation. Whether an aged microbiota may alter the host pre-existing microflora is still an open question. Therefore, this study aimed to elucidate the impact of the age of donor microbiota provided by healthy wild type C57BL/6 mice in young AD transgenic mice over a defined period. As a starting point, 5XFAD model mice were treated with antibiotics to induce a depletion of the existing microbial communities and later, with cecal suspension provided either from age-matched "young" or mid-age "old" wild type mice. Then, several features, such as the microbial composition, mice behavior and *post-mortem* pathological hallmarks were evaluated. The results shown in the following chapters (from 4.2 to 4.7) have been published in Valeri et al. (2021) and Nguyen et al. (2021).
Chronic stress exposure represents another contributing factor implicated in AD development. In particular, stress has been associated to exacerbation of the pathogenesis in rodents as observed by increased acceleration in AD hallmarks (see review Justice, 2018). In the last decade, stress has been connected to the gut dysbiosis and to related processes at the base of the intestinal permeability and finally, to the establishment of a "leaky gut". Moreover, stress may exert its function on the ENS through the GBA. The capacity of mice to adapt to stress maintaining their homeostasis is defined as resilience, while the opposite as susceptibility (Russo et al., 2012; Rutter, 1993; Southwick et al., 2005). In the here presented second study, male and female Arc-sfGFP mice aged 4 weeks were subjected to chronic social defeat stress (CSDS) paradigm as described elsewhere (Dos Santos Guilherme et al., 2022) to allow a stratification in resilient and susceptible mice. Then, the tamoxifen-induced neuronal activation was evaluated in the ileum portion of the small intestine *myenteric plexus* between stressed (susceptible and resilient mice) and unstressed control mice. The results have been published in Dos Santos Guilherme et al. (2022) and are described in chapter 4.. All unpublished results are referred to in the respective figure legend.

## 4.1 The effect of aged cecal microbiota transfer from healthy wild type into 5XFAD mice

A detail description of the effects of cecal transplant from healthy wild type donors into transgenic 5XFAD mice was provided in the following chapters (from 4.2 to 4.7) and the results behind were published in Valeri et al. (2021) and Nguyen et al. (2021). The timeline adopted for the experimental procedure was graphically represented in figure 7A. FMT-treated 5XFAD mice were subjected to a battery of behavioral tasks within the last two weeks before sacrifice as shown in figure 7B.

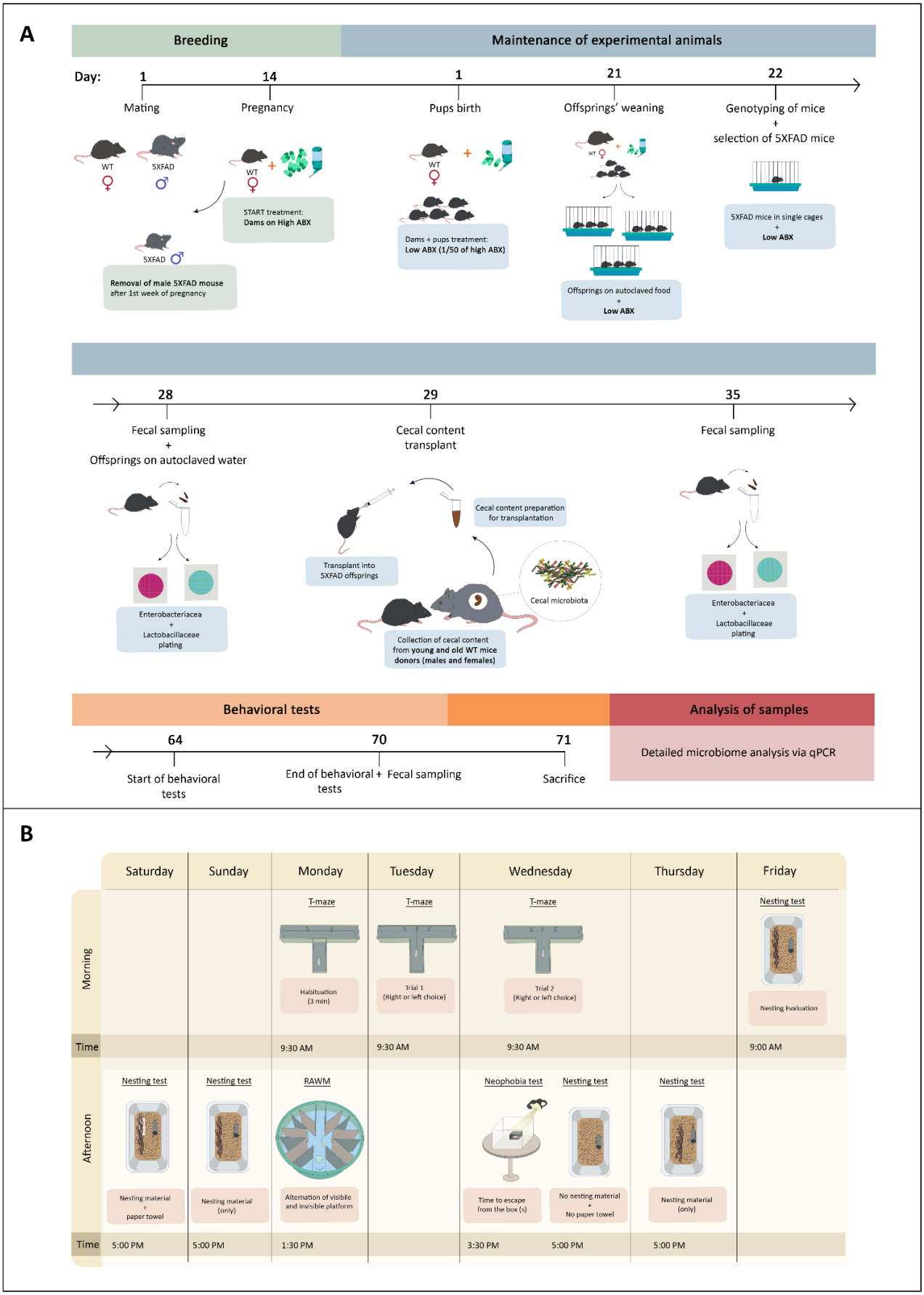

A
Breeding
Maintenance of experimental animals
Day:
1
14
1
21
22
Mating
Pregnancy
Pups birth
Offsprings' weaning
Genotyping of mice + selection of 5XFAD mice
WT
5XFAD
START treatment: Dams on High ABX
Removal of male 5XFAD mouse after 1st week of pregnancy
Dams + pups treatment: Low ABX (1/50 of high ABX)
Offsprings on autoclaved food + Low ABX
5XFAD mice in single cages + Low ABX
28
29
35
Fecal sampling + Offsprings on autoclaved water
Cecal content transplant
Fecal sampling
Enterobacteriacea + Lactobacillaceae plating
Cecal content preparation for transplantation
Transplant into 5XFAD offsprings
Cecal microbiota
Collection of cecal content from young and old WT mice donors (males and females)
Enterobacteriacea + Lactobacillaceae plating
Behavioral tests
Analysis of samples
64
70
71
Start of behavioral tests
End of behavioral tests + Fecal sampling
Sacrifice
Detailed microbiome analysis via qPCR
B
Saturday
Sunday
Monday
Tuesday
Wednesday
Thursday
Friday
Morning
T-maze
Habituation (3 min)
Trial 1 (Right or left choice)
Trial 2 (Right or left choice)
Nesting test
Nesting Evaluation
Time
9:30 AM
9:30 AM
9:30 AM
9:00 AM
Afternoon
Nesting test
Nesting material + paper towel
Nesting material (only)
RAWM
Alternation of visible and invisible platform
Neophobia test
Time to escape from the box (s)
No nesting material + No paper towel
Nesting material (only)
5:00 PM
5:00 PM
1:30 PM
3:30 PM
5:00 PM
5:00 PM

**Figure 7: Graphical representation of the experimental procedure.** (A) Wild type (WT) females were mated with male transgenic 5XFAD mice (day 1). At the second week of pregnancy (day 14), dams were administrated high dose of antibiotics treatment (high ABX). Antibiotics mixture was further reduced to 2/3 of high ABX in the last week before pups' birth. At the mice pups' birth (P1), antibiotics were reduced (1/50 of the original dosage, low ABX). 5XFAD mice pups were weaned and single caged at 3 weeks of age (P21) and kept at low dosage ABXs treatment until the fourth week (P28). At P28, ABXs were replaced with autoclaved tap water in order to remove the ABXs residues in the mice intestine one day before the transplant of the donors' caecum content. Fecal material was collected from recipients at different time points: at P28, the day right before fecal transfer, at P35, 1 week after the fecal transplant and at P70, 6 weeks after the fecal transfer. (B) 5XFAD mice underwent a scheduled-planned battery of behavioral tasks from P64 to 70. Mice were sacrificed at P71 and material for molecular analysis collected. The graphical representation of the experimental procedure was slightly modified from Valeri et al. (2021). Abbreviations: ABX (antibiotics), high ABX (high antibiotics concentration), low ABX (low antibiotics concentration, 1/50 of the original dosage), P (postnatal), qPCR (quantitative PCR), 5XFAD (Alzheimer's disease mouse model), WT (wild type).

## 4.2 Analysis of gut microbiota composition in wild type donors

The choice of the donor mice target age for this investigation was based on recent reports (Dutta and Sengupta, 2016; Flurkey et al., 2007; Shoji et al., 2016). A large-scale study conducted by Shoji et al. (2016) on a total of 1739 C57BL/6 mice revealed behavioral differences between the investigated age groups (2–3, 4–5, 6–7, and 8–12-month-old) that may be directly compared to different phases of human development. In particular, the 8–12-month-old mice group showed more pronounced changes in most of the behaviors observed (i.e., decreased locomotor activity to novel environments, motor function, acoustic startle response, social behavior, and depression-related behavior, increased prepulse inhibition, and deficits in spatial and cued fear memory), and increased anxiety-like behavior in the light/dark test when compared to the other age groups (Shoji et al., 2016). Dutta and Sengupta (2016) provided a calculation to derive the mouse age from humans considering the total lifespan of the animals (Dutta and Sengupta, 2016). In particular, mice aged around 12 months are considered being equivalent to humans with age range 38-55 years old (Flurkey et al., 2007). In addition, as already reported in the previous chapters, C57BL/6 mice from 12 month of age and upwards exhibited impairments in spatial learning and memory functions that may be similar to the ones observed in humans aged between 60-70 years old (Barrash, 1994; Shoji et al., 2016). Donor mice aged 1 month were comparable to human adolescents (between 12 and 14 years old) and to 5XFAD mice before the onset of AD symptoms and pathological changes (i.e., soluble Aβ starts to accumulate in the brain around 1.5 months of age) (Flurkey et al., 2007; Oakley et al., 2006).
Wild type donors were investigated with selective plates for Enterobacteriaceae and Lactobacillaceae growth and by qPCR for assessing specific groups of bacteria of mice aged 1 month (designated as "young") and 12 months (designated as "old"). The choice of Enterobacteriaceae and Lactobacillaceae as representative families for such investigation was based mainly on their opposite role on the host health (see chapter 1.9). Indeed, while most of the Lactobacillaceae strains are probiotics or beneficial commensals that have been associated with amelioration of the human mood and depression states (Akkasheh et al., 2016; Benton et al., 2007), Enterobacteriaceae mostly include pathogenic bacterial species often associated to gut inflammation (Pédron and Sansonetti, 2008). Sex is an essential variable to consider in the experiment design for its relation to AD and to aging (see chapter 1.3). Recently, several studies showed that sex differences play an essential role in relation to the gut microbiota composition (see chapter 1.7.3). Therefore, an equal number of mice of both sexes were used for the fecal microbiota analysis to avoid biased results. The importance of sex in relation to gut microbiota and age was already discussed in the published review by Valeri and Endres (2021). In the following next two subchapters the changes in donors' Enterobacteriaceae and Lactobacillaceae levels (subchapter 4.2.1) and further bacterial groups (subchapter 4.2.2) are described.

### 4.2.1 Analysis of Enterobacteriaceae and Lactobacillaceae levels between young and old wild type mice donors

Firstly, Enterobacteriaceae and Lactobacillaceae were analyzed in fecal samples from wild type (C57BL/6J) mice donors at the two different age points of 1 month (designated as "young") and 12 months (designated as "old") by counting bacterial colonies (CFUs/mg) on selective microplates. Old wild type mice (*n* = 10) showed significantly increased levels of both Enterobacteriaceae and Lactobacillaceae compared to young mice (*n* = 8). Lactobacillaceae amount was found two-fold higher in old mice compared to young mice (*p* = 0.0003), while Enterobacteriaceae levels were nearly three-fold higher in old mice compared to young mice (*p* = 0.048) (Fig. 8). These results are consistent with previous reports that identified increased levels of Enterobacteriaceae and Lactobacillaceae species in human elderly (aged > 60 years) compared to younger adults (aged between 20-50 years) (chapter 1.9; Table 2; Mueller et al., 2006; Rahayu et al., 2019). In addition, the elevation of Enterobacteriaceae was also observed in the fecal microbiome of aged (21–22 months) mice but not in the young (4–5 months) mice after *S. pneumoniae* infection (McMahan et al., 2022). Alteration in Lactobacillaceae levels due to aging was also observed by Langille et al. (2014) in female C57BL/6J mice. In this case, higher levels of Lactobacillaceae were obtained in the younger (174 ± 15 days old) group of female mice compared to older groups (middle: 589 ± 18 days old; old: 857 ± 16 days old). Such divergence in the Lactobacillaceae levels may be explained with a different methodology used (CFUs counting on selective plates versus PCR analysis), sex differences (half males/females versus only females) and non-comparable different groups of age (above 2 years versus 12 months for the old group and mean of 6,3 months versus 1 month for the young group).

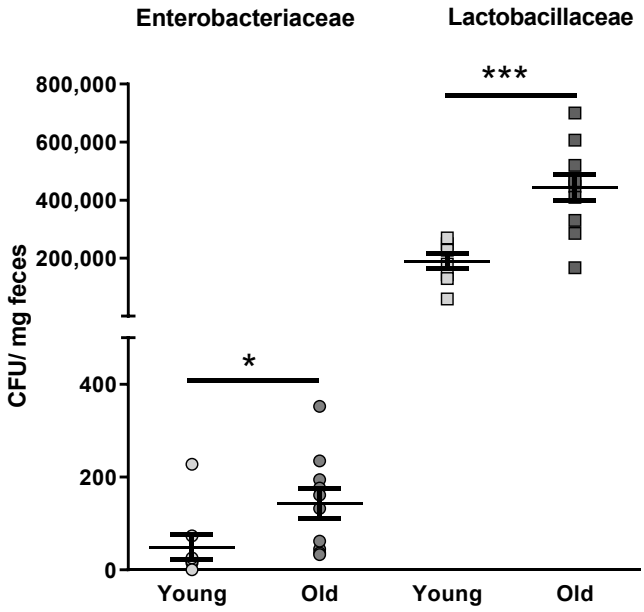
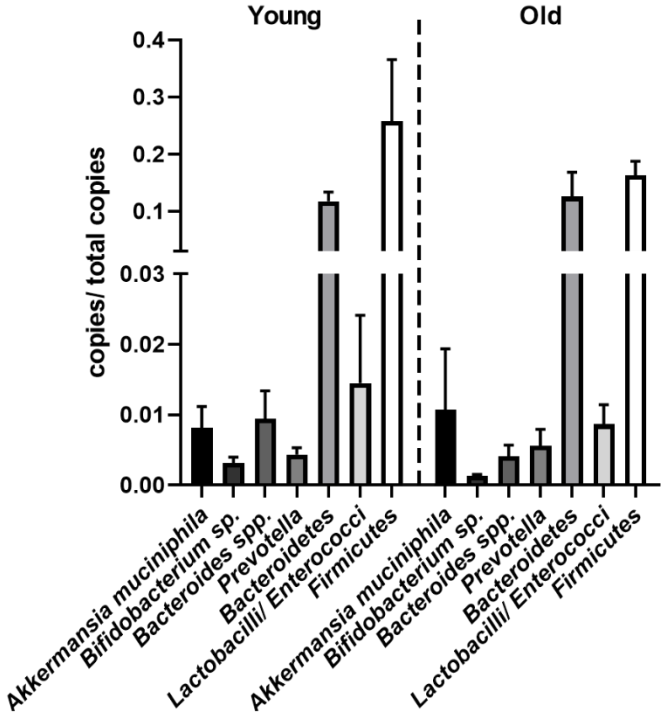



**Figure 8: Assessment of viable Enterobacteriaceae and Lactobacillaceae in young and old wild type mice donors.** Fecal samples from wild type mice were collected at 1 month (designated as young) and 12 months (designated as old) of age (*n* = 8 for young; *n* = 10 for old), diluted with sterile NaCl 0.9% and plated in a sample-ready-culture-medium system (3M™ Petrifilm™) for specific Enterobacteriaceae and Lactobacillaceae growth. Colony forming units (CFUs) per mg were counted after 24 h of incubation at 37°C. Data are presented as the mean ± SEM. Statistical analysis was performed by One Way ANOVA with Fisher's LSD test (*, $p < 0.05$; ***, $p < 0.001$). Data were published in Valeri et al. (2021). Abbreviations: Young (1-month wild type donors), Old (12-months wild type donors), CFU (Colony forming units).

### 4.2.2 Analysis of specific group of bacteria by qPCR in wild type donors

Microbial DNA contained in fecal samples of wild type donors was also investigated by qPCR for a more complete overview of the groups of microbial bacteria involved. Such analysis was conducted at the MVZ Institut fuer Mikrooekologie GmbH (Herborn, Germany) and the procedure behind was described elsewhere (Brandscheid et al., 2017; Valeri et al., 2021).
*Akkermansia muciniphila* (Phylum Verrucomicrobia) is a highly abundant (about 3%) bacterial species in the human colon, associated with a healthy gut in early-middle and late-middle age (de Vos, 2017). Some studies showed a decline of *Akkermansia muciniphila* with age in humans (Collado et al., 2007) and in mice (Fransen et al., 2017; Langille et al., 2014; van der Lugt et al., 2018), while only another study showed increased abundance of *Akkermansia muciniphila* in the elderly (Biagi et al., 2010) and in centenarians (105–109 years old) compared to younger age groups (Biagi et al., 2016). Concerning *Bacteroides* and *Prevotella* (Phylum Bacteroidetes), a significant reduction was reported in elderly and individuals with high frailty scores (Saraswati and Sitaraman, 2014; Zwielehner et al., 2009). Contrarily to the previous reports, in the here presented study, no differences in *Akkermansia muciniphila* species, *Bacteroides* and *Prevotella* genera and in *Lactobacilli/Enterococci* ratio due to aging were observed. Such different outcome may be explained with several factors involved, such as different mice strains and the use of mice with different age. In this study, mice were aged 12 months and potentially may be considered equivalent to human adults with age range 38-55 years old (Flurkey et al., 2007). Based on that assumption, 12-month-old mice may be considered too young for a direct comparison with the previously reported studies on human elderly or older mice. Therefore, lack of differences in the target groups of bacteria is highly plausible. However, concerning the general Firmicutes phylum, a significant decrease was observed in the old group compared to the young one (Fig. 9).




| | young-> old | p-value | n young/old |
|---|---|---|---|
| *Akkermansia muciniphila* | ⦸ | 0.946 | 8/5 |
| *Bifido-bacterium sp.* | ⦸ | 0.958 | 7/7 |
| *Bacteroides spp.* | ⦸ | 0.870 | 8/8 |
| *Prevotella* | ⦸ | 0.974 | 8/5 |
| *Bacteroidetes* | ⦸ | 0.823 | 8/6 |
| *Lactobacilli/ Enterococci* | ⦸ | 0.859 | 8/8 |
| *Firmicutes* | ⇩ | 0.008 | 6/8 |

**Figure 9: Assessment of different groups of bacteria in healthy untreated wild type mice donors.** Fecal material was collected in healthy untreated wild type mice. Genomic DNA was assessed by qPCR with specific primer pairs for detecting different groups of bacteria. Analysis conducted at the MVZ Institut fuer Mikrooekologie GmbH (Herborn, Germany). A graphic representation of the investigated groups of bacteria between young and old donors is given on the left, while p-value, numbers of mice investigated and difference between young and old mice were shown in the right table. The arrows pointing down represent a significant decrease in the amount of the defined bacteria in the old group. Data are presented as the mean + SEM. Statistical analysis was performed by One Way ANOVA corrected with Fisher's LSD test (*, $p < 0.05$; **, $p < 0.01$). Data and methods were published in Valeri et al. (2021). Abbreviations: Young (wild type young donors), Old (wild type old donors).

## 4.3 Fecal microbiota transplant procedure in 5XFAD mice

Alteration in microbial composition was assessed for certain groups of bacteria in relation to the aging process in healthy wild type mice (see previous chapters 3.1.1 and 3.1.2). Then, antibiotics-pre-treated transgenic 5XFAD mice were inoculated with cecal content from either young or old wild type donors. The entire procedure is described in Valeri et al. (2021) and in methods section (from chapter 2.2.2 to 2.2.4) (Fig. 7A). This includes a first step in which, dams and transgenic 5XFAD offsprings mice were administrated with antibiotics (subchapter 4.3.1) and, a second step, in which transgenic 5XFAD mice were inoculated with diluted cecal content from young or old wild type donors (subchapter 4.3.2).

### 4.3.1 Antibiotic treatment efficiency in dams and recipient 5XFAD offspring mice

Prior to the FMT inoculation, recipient 5XFAD mice were pre-treated with a cocktail of antibiotics to severely reduce the amount of the pre-existing bacteria residing in the gut and to allow the colonization of the donor microbiota. Efficient antibiotics experimental procedure was already described by Minter et al. (2016) in 6-months old APPswe/PS1ΔE9 mice. Antibiotics dosage was adapted to transgenic 5XFAD mouse model taking into consideration their early age of administration (prenatal treatment until 3 weeks of age versus treatment at 6 months of age) and the route of administration (drinking water versus gavage). Intergenerational transmission of bacteria from dams to pups has been observed during pregnancy both in Wistar rats and C57BL/6 mice (Schulfer et al., 2018; Tulstrup et al., 2018). Therefore, to improve the efficiency of the treatment in the newborn pups, the antibiotics mixture was already administrated during the dams' last trimester of pregnancy. Moreover, antibiotic procedure was adapted from Minter et al. (2016) removing kanamycin recipe for its potential teratogenic effect on the litters' health (Holdiness, 1987) and reducing the reagent concentrations (2/3 of the initially described dosages) in the last week of pregnancy before giving birth. In addition, to avoid, or at least, to reduce any possible stress to the dams and offsprings' caused by daily chronic oral gavage, antibiotics were administered *ad libitum* in the drinking bottle as described elsewhere (C. S. Wu et al., 2021). Dams' fecal samples were collected 1 day after the pups weaning to avoid any possible relevant stress during the collection phase. The efficiency of antibiotics treatment in dams ($n$ = 9) was proven by a significant reduction of both the examined representative bacteria families in comparison to untreated aged-matched dams ($n$ = 9). In detail, Lactobacillaceae family showed a significant reduction by factor 62 ($p$ = 0.0012), while Enterobacteriaceae were significantly reduced by factor 43 ($p$ = 0.046) (Fig. 10).

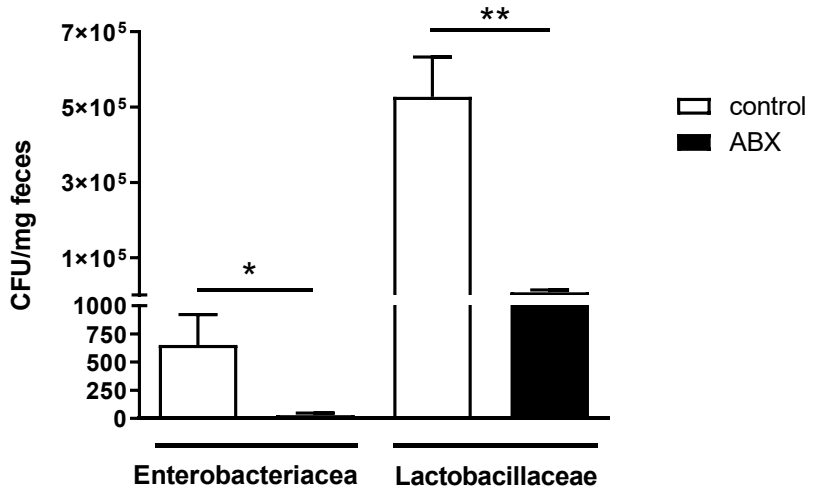



**Figure 10: Assessment of viable Enterobacteriaceae and Lactobacillaceae in treated and untreated dams.** To avoid any causative stress to the pups during the sample collection, dams' fecal samples were collected in the last week of pregnancy. Then, fecal samples from dams ($n$ = 9 per group) were diluted with sterile NaCl 0.9% and plated in a sample-ready-culture-medium system (3M™ Petrifilm™) for the Enterobacteriaceae and Lactobacillaceae growth. Colony forming units (CFUs) per mg were counted after 24 h of incubation at 37°C. Data are presented as the mean + SEM. Statistical analysis was performed by One Way ANOVA with Fisher's LSD test (*, $p < 0.05$; **, $p < 0.01$). Data are published in Valeri et al. (2021). Abbreviations: ABX (antibiotics' mixture), control (untreated dams), CFU (Colony forming units).

At the pups' birth, antibiotics concentration was further reduced to 1/50 (low dose; as previously described by Minter et al., 2016) of the original dose and administrated until the day before the cecal inoculation (around P28). This is an important factor to consider as the original higher dose used for the dams may be deleterious for the newborns or might affect postnatal development. Hence, litter size was daily monitored for the entire duration of the antibiotics' treatment. Although antibiotic-treated offspring mice showed a significant increase in the mean litter size compared to untreated control mice (*, $p = 0.027$), no differences were observed in their sex ratio (Table 4). In addition, daily health and welfare care check did not show any adverse effect of antibiotics treatment both on pregnant mothers and pups (health evaluation of experimental laboratory mice described by Burkholder et al. (2012)).

| | Littersize | | n | Sex ratio | |
|---|---|---|---|---|---|
| | mean | SD | | mean | SD |
| control | 6,10 | 2,34 | 21 | 1,38 | 1,31 |
| ABX | 7,57* | 1,78 | 21 | 1,58 | 1,00 |

**Table 4: Litter features.** Mice litter from antibiotics-treated (ABX) and control dams (control) giving birth in the same period were compared. Statistical analysis was performed by using Student's t-test (*, $p < 0.05$). Data are published in Valeri et al. (2021). Abbreviations: ABX (antibiotics), control (untreated mice), SD (Standard deviation), $n$ (number of mice).

The efficiency of the antibiotics' chronic exposition (started already during dams' pregnancy) was evaluated 1 week after weaning in the transgenic 5XFAD offspring mice. Antibiotics-treated mice (*n* = 38) showed a significant reduction of both Enterobacteriaceae and Lactobacillaceae compared to control untreated mice (*n* = 18) demonstrating the efficacy of the treatment. In particular, Lactobacillaceae family showed a significant reduction by factor 2 (*p* = 0.002), while Enterobacteriaceae showed a significant reduction by factor 6 (*p* = 0.049) (Fig. 11). However, efficiency was weaker, but still statistically significant, as observed in antibiotics-treated dams.

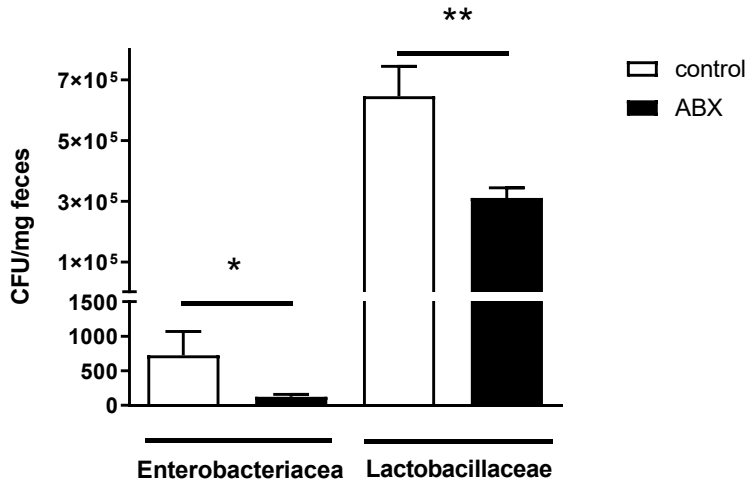



**Figure 11: Assessment of viable Enterobacteriaceae and Lactobacillaceae in antibiotic-treated and untreated 5XFAD offsprings mice.** Fecal samples from antibiotics-treated (*n* = 38) and control untreated (*n* = 18; receiving normal drinking water without antibiotic supplements) 5XFAD mice were collected 1 week after weaning, diluted with sterile NaCl 0.9% and plated in a sample-ready-culture-medium system (3M™ Petrifilm™) for the Enterobacteriaceae and Lactobacillaceae growth. Colony forming units (CFUs) per mg were counted after 24 h of incubation at 37°C. Data are presented as the mean + SEM. Statistical analysis was performed by One Way ANOVA and corrected with Fisher's LSD test (*, $p < 0.05$; **, $p < 0.01$). Data are published in Valeri et al., 2021. Abbreviations: ABX (antibiotics), control (untreated 5XFAD offsprings mice), CFU (Colony forming unit).

The use of antibiotics or germ-free mice has already been discussed regarding their effects on host physiology, including cellular composition, signaling pathways, and organ function (Kennedy et al., 2018). Both approaches have strengths and weaknesses also due to their differences in action mechanisms. Many aspects of development and early immune education are broadly impaired in germ-free mice. This includes the gut barrier permeability and behavioral aspects. Indeed, anxiety-like phenotype observed in germ-free mice may be the result of the imbalances of the HPA axis caused by intestinal microbes (Hayes et al., 2018; Huo et al., 2017; Luczynski et al., 2016). On the contrary, antibiotics treatment can be applied to any genotype and this - especially when using non-systemic antibiotics - allows the maintenance of cell functionality and signaling pathways after development. In addition, the use of a broad spectrum of antibiotics may be considered as the easier approach for a fast assessment of bacterial depletion. This can be carried out by counting bacterial CFUs from fecal samples plated in aerobic and/or anaerobic conditions on non-selective media (Kennedy et al., 2018). However, a complete depletion of bacteria is not completely guaranteed by antibiotics treatment as also observed in this study. Comparable outcomes were described with germ-free mice, such as significant reduction in bacterial load associated with shifts in cell populations, signaling pathways, and organ morphology (Kennedy et al., 2018). On one hand, the use of a wide spectrum antibiotics showed positive results in APPswe/PS1ΔE9 mice, including reduction of Aβ plaques deposition and

neuroinflammation in aged (6 months) mice (Minter et al., 2016). On the other hand, long-term perturbations in microbial diversity and genera (principally Lachnospiraceae and S24-7) were induced in APPswe/PS1ΔE9 mice after being exposed to early postnatal antibiotics treatment (P14-P21) (Minter et al., 2017). Several studies displayed those metabolic pathways involved in AD-related diseases, including diabetes, might be affected by some antibiotics (e.g., vancomycin, bacitracin and metronidazole). Soto et al. (2018) revealed alteration of metabolic homeostasis with improvement of insulin signaling in the brain and reduction of anxiety and depression behaviors in mice after treatment with vancomycin or metronidazole antibiotics (Soto et al., 2018). Zarrinpar et al. (2018) showed that C57BL/6 mice treated with a cocktail of antibiotics (ampicillin, vancomycin, metronidazole, neomycin, and amphotericin B) alter the gut homeostasis, the luminal signaling and metabolism decreasing baseline serum glucose levels, reducing glucose surge in a tolerance test, and improving insulin sensitivity without altering adiposity (Zarrinpar et al., 2018). Similarly, treatment of C57BL/6 mice with vancomycin and bacitracin antibiotics ameliorated simultaneously, hyperinsulinemia, systemic glucose intolerance and insulin resistance (Hwang et al., 2015). Chronic treatment (for 14 weeks) with a mixture of antibiotics (i.e., gentamicin, vancomycin, metronidazole, neomycin, ampicillin, kanamycin, colistin, and cefoperazone) reduced plaque load in the hippocampus and improved nesting score in 5XFAD mice (Dos Santos Guilherme et al., 2021). In addition, while antibiotics did not affect various of the physiological parameter tested, blood sugar and serum glucagon levels were affected (Dos Santos Guilherme et al., 2021). Another recent study showed that antibiotics treatment, similar to the ones used in the presented study, seem not to be able to reach the brain in APPswe/PS1ΔE9 mice (Dodiya et al., 2020). In addition, early life exposure to maternal antibiotics influences the offspring behavior (e.g., anxiety, sociability and cognitive behaviors) (O'Connor et al., 2021; Tochitani et al., 2016). Altogether, these studies contribute to the assumption that, despite some pitfalls, antibiotics exert a beneficial effect on mice health especially concerning diabetes-like phenotype. Here, efficiency of antibiotics administration was confirmed with a drastic reduction of selected target bacteria in both treated pregnant mothers and 5XFAD offsprings. This provides a useful platform for the inoculation of the donors' microbial communities in recipient 5XFAD mice.

### 4.3.2 FMT treatment of 5XFAD mice

Wild type mice donors of both sexes were dissected in equal amount (half males/females), and the material contained in the caecum region of the intestine collected, diluted accordingly, and stored at -80 °C until the day of inoculation (maximum storage of 1 month). Therefore, donors' caecum content was collected within 1 month before the FMT in order to preserve the intact properties of the microbiota composition as described elsewhere (Gweon and Na, 2021). The used FMT parameters described in this study were based on Ellekilde et al. (2014). Indeed, a successful establishment of the donor microbiota in recipient C57BL/6 mice was already demonstrated in mice around the third week of age (Ellekilde et al., 2014). Here, 5XFAD mice at P28 (1 week after weaning) were subjected to a single acute administration of FMT by oral gavage. Prior the inoculation, 5XFAD mice were divided in two groups, "young" and "old", based on their future assigned treatment. As initial step, baseline levels of Enterobacteriaceae and Lactobacteriaceae were addressed in mouse groups after antibiotics administration and right before being subjected to FMT. No differences were detected between the assigned groups concerning Enterobacteriaceae ($p$ = 0.9996) and Lactobacteriaceae ($p$ = 0.6594) levels, confirming the presence of microbial homogeneity (Fig. 12).

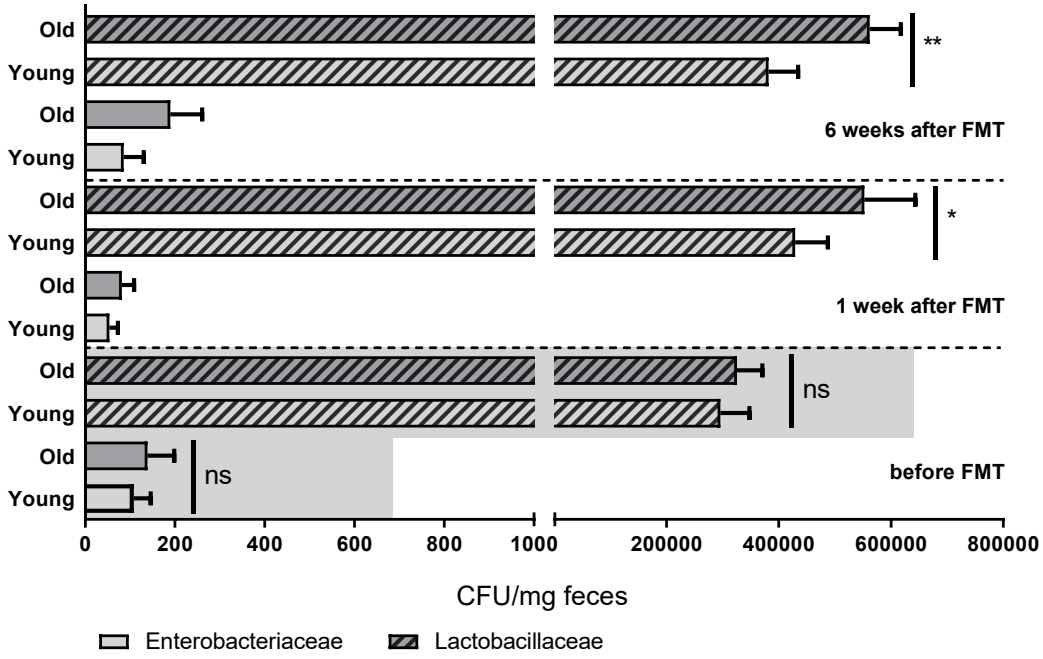



**Figure 12: Assessment of viable Enterobacteriaceae and Lactobacteriaceae in recipient FMT-treated 5XFAD mice.** Fecal material was collected at different time points: right before FMT and after 1 week and 6 weeks from FMT in 5XFAD mice (*n* = 20 per group, 10 for both females and males). Then, fecal material was diluted with sterile NaCl 0.9% and plated in a sample-ready-culture-medium system (3M™ Petrifilm™) for the Enterobacteriaceae and Lactobacillaceae growth. 5XFAD mice were designated as young (1 month) and old (12 months) based on the received treatment from wild type donors by oral gavage. Colony forming units (CFUs) per mg were counted after 24 h of incubation at 37°C on selective plates for Enterobacteriaceae and Lactobacteriaceae families. Data are presented as the mean + SEM. Statistical analysis was performed by One Way ANOVA and corrected with Fisher's LSD test (*, $p < 0.05$; **, $p < 0.01$). CFUs/mg values of mice not subjected to antibiotics treatment were represented by the height of grey boxes. Data are published in Valeri et al., 2021. Abbreviations: Young (treatment with cecal content from young wild type donors), Old (treatment with cecal content from old wild type donors), FMT (cecal material transplant), ns (not statistically significant), CFU (Colony forming unit).

To define the impact of FMT in the early phase of development (1 week after transplantation) and in later stages (6 weeks after transplantation), Enterobacteriaceae and Lactobacteriaceae levels were compared between aged-matched 5XFAD mice that received caecum content from young donors (termed as 5XFAD + FMT young) and mock (treated with PBS1X, termed as 5XFAD – FMT) treatment. No differences in the mentioned target bacterial families were detected between the assigned groups, suggesting a null effect of the microbiota transplant from young wild type donors, comparable to a lack of treatment, in both its early and later phase of development (Fig. 13 A and B).

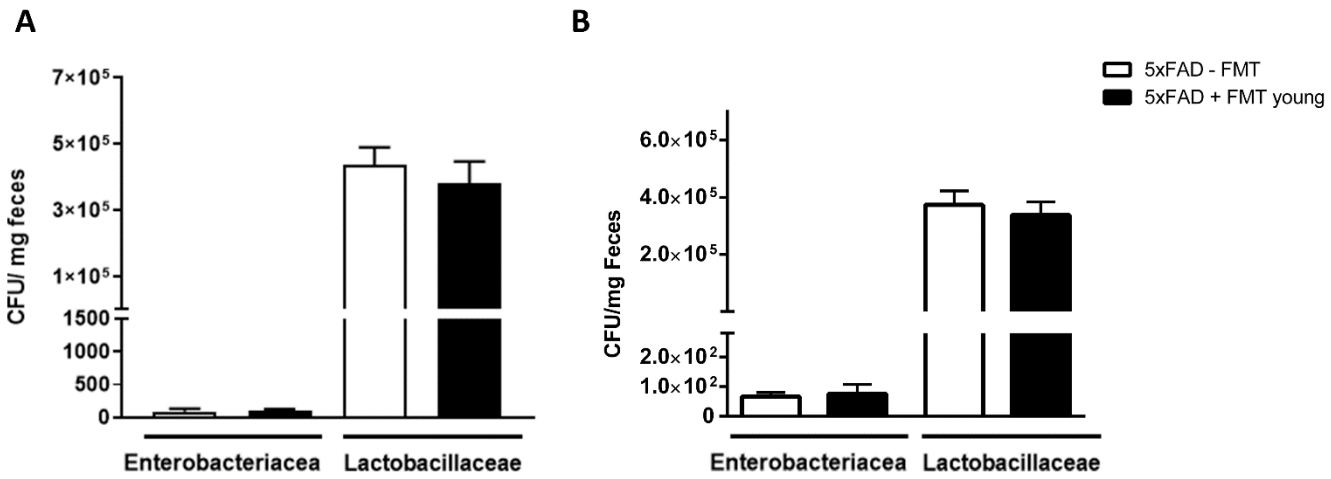
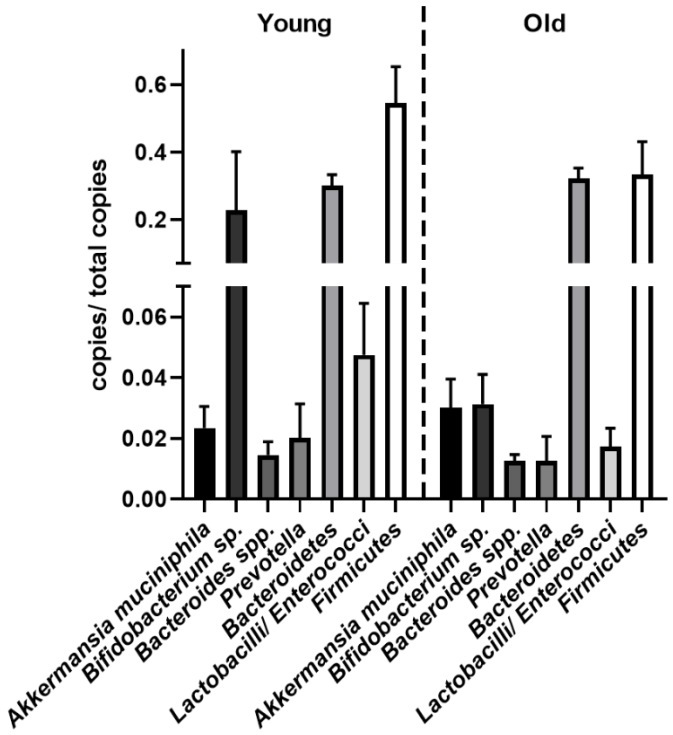



**Figure 13: Assessment of viable Enterobacteriaceae and Lactobacteriaceae in aged-matched 5XFAD offspring mice subjected to FMT from young wild type donors or to mock treatment**. Suspended and diluted fecal material was analyzed and the colony forming units (CFUs) per mg counted after 24 h of incubation at 37°C on selective plates for Enterobacteriaceae and Lactobacteriaceae families. Animals were either treated with mock solution (treated with PBS1X, 5XFAD - FMT) or with caecum fecal content from age-matched young donors (5XFAD + FMT young). (A) Assessment after 1 week: *n* = 15 for 5XFAD – FMT and *n* = 20 for 5XFAD + FMT young. (B) Assessment after 6 weeks: *n* = 14 (Enterobacteriacea), *n* = 15 (Lactobacteriaceae) for 5XFAD – FMT; *n* = 15 (Enterobacteriacea), *n* = 18 (Lactobacteriaceae) for 5XFAD + FMT young. Statistical analysis was performed by using Student's t-test (*, $p < 0.05$). Data are presented as the mean + SEM. (A) Data published in Valeri et al., 2021; (B) unpublished data. Abbreviations: 5XFAD – FMT (5XFAD mice treated only with PBS1X), 5XFAD + FMT young (5XFAD mice treated with cecal content provided by young healthy wild type donors), CFU (colony forming unit**)**.

Then, the effect of the microbiota transplant was investigated in 5XFAD mice treated with cecal content from young and old wild type donors at the two target points of 1 week and 6 weeks after the oral gavage (Fig. 12). Interestingly, transgenic 5XFAD mice receiving material from old donors displayed significantly increased Lactobacillaceae levels at both 1 and 6 weeks after treatment in comparison to the ones that received material from young donors (Fig. 12). Concerning the Enterobacteriaceae, 1 week after FMT, higher CFUs mean count was detected in the 5XFAD mice treated with caecum content from old donors (86 versus 40 CFU/mg) but without reaching statistical significance. After 6 weeks from FMT, a tendency to increase was registered in the old group concerning Enterobacteriaceae count (44 versus 200 CFU/mg) but the p-value remained below significance level ($p = 0.0664$) (Fig. 12).

To have a more wide and complete spectrum analysis of the bacteria species affected by FMT, the genomic DNA (gDNA) of the fecal material was also analyzed by qPCR in FMT-treated 5XFAD mice (young versus old) (Fig. 14). Such analysis was conducted at the MVZ Institut fuer Mikrooekologie GmbH (Herborn, Germany) and the procedure behind was described elsewhere (Brandscheid et al., 2017; Valeri et al., 2021). However, a direct comparison of bacterial counts between 5XFAD (Fig. 14) and wild type mice groups (Fig. 9) is not feasible due to lack of antibiotics treatment in the latter. On one hand, both FMT-treated 5XFAD and untreated wild type mice donors did not show differences in *Akkermansia muciniphila* species (Phylum Verrucomicrobia), *Bacteroides* and *Prevotella* genera (Phylum Bacteroidetes) and in *Lactobacilli*/*Enterococci* ratio due to aging. On the other hand, a significant robust lower amount in general Firmicutes phylum levels was displayed in the old groups (both FMT-treated 5XFAD and untreated wild type mice donors) in comparison to their respective young groups ($p = 0.008$ for wild type mice donors and $p = 0.006$ for treated 5XFAD mice). A significant lower amount was also observed for the *Bifidobacterium* genus (Phylum Actinobacteria) in recipient 5XFAD mice treated with cecal material from old wild type mice ($p = 0.011$; 0.229 counts versus 0.031 counts/total counts) (Fig. 14), while it did not get statistically significant ($p = 0.96$; 0.003 counts versus 0.001 counts/total counts) in untreated aged wild type mice (Fig. 9)




| | young-> old | p-value | n young/old |
|---|---|---|---|
| *Akkermansia muciniphila* | ⦸ | 0,941 | 14/15 |
| *Bifidobacterium sp.* | ⇩ | 0,011 | 20/20 |
| *Bacteroides spp.* | ⦸ | 0,979 | 20/20 |
| *Bacteroidetes* | ⦸ | 0.255 | 17/20 |
| *Prevotella* | ⦸ | 0,960 | 8/4 |
| *Lactobacilli/ Enterococci* | ⦸ | 0,699 | 20/19 |
| *Firmicutes* | ⇩ | 0,006 | 20/20 |

**Figure 14: Assessment of different groups of bacteria in FMT-treated recipient 5XFAD mice by qPCR.** Fecal material was collected in FMT-treated recipient 5XFAD mice after 6 weeks from cecal inoculation. Genomic DNA was assessed by qPCR with specific primer pairs for detecting different groups of bacteria. Analysis conducted at the MVZ Institut fuer Mikrooekologie GmbH (Herborn, Germany). A graphic representation of the investigated groups of bacteria between young and old FMT-treated 5XFAD mice is given on the left, while p-value, numbers of mice investigated and difference between young and old mice were shown in the right table. The arrows pointing down represent a significant decrease in the amount of the defined bacteria in the old group. Data are presented as the mean + SEM. Statistical analysis was performed by One Way ANOVA corrected with Fisher's LSD test (*, $p < 0.05$; **, $p < 0.01$). Data and methods are published in Valeri et al. (2021). Abbreviations: Young (treatment with cecal content from young wild type donors), Old (treatment with cecal content from old wild type donors).

In summary, the transfer of caecum microbiota was able to reproduce the differences in Enterobacteriaceae and Lactobacillaceae CFUs/mg, observed in wild type donors due to aging process, into recipient 5XFAD mice up to 6 weeks from the oral gavage (Fig. 12). As already mentioned before (chapters 1.9.1, 1.9.2 and 4.2), the Enterobacteriaceae and Lactobacillaceae were used as representative families within the microbial community for their role in relation to aging. In particular, increased levels of Lactobacillaceae were observed both at 1 week and 6 weeks after treatment. Contrarily, no significative differences in Enterobacteriaceae levels were observed between treatments despite for a slight tendency to increase in the old group at 6 weeks after treatment (Fig. 12). Moreover, analysis of microbial species evaluated by qPCR were supported by previously described studies (see chapter 1.9 and Table 2) in which, a diminished Firmicutes to Bacteroidetes ratio, has been associated to human adults and elderly (> 60 years). Similar results (showing higher level of Firmicutes/Bacteroidetes ratio) were also shown in 6-month-old mice, while older age groups showed a decline in Firmicutes levels (C. S. Wu et al., 2021). In accordance with already reported studies (summarized in Table 2), decreased level of *Bifidobacteria* were also observed in the here presented study in 5XFAD mice treated with cecal content from old donors compared to ones treated with young cecal content (Fig. 14). Wild type donor mice did not show such difference in *Bifidobacteria* levels among young and old groups (Fig. 9). However, a small number of wild type mice was used for this investigation suggesting that, lack of comparable numbers between transgenic and wild type mice ($n = 20$ for FMT treated-5XFAD mice and 7 for wild type mice donors), may interfere with the described outcomes. Therefore, it may be plausible that an enlarged group of wild type mice may generate sufficient statistical power for the investigated subgroups of bacteria, including *Bifidobacteria*.

## 4.4 Behavioral task performance in FMT-treated recipient 5XFAD mice

Among the AD transgenic mouse models, 5XFAD mice start to exhibit impairment in memory (e.g., learning and memory deficits) and anxiety- and depressive-like behaviors at approximately 4 to 5 months of age (Devi and Ohno, 2010; Locci et al., 2021; Oakley et al., 2006; see chapter 1.5). Physiologic behaviors, including mood and cognition, can be modulated by gut microbiota as observed by increased anxiety and depression-like behaviors in germ-free and special pathogen-free rats lacking of gut microbiota or with a dysregulation of HPA (Crumeyrolle-Arias et al., 2014). In addition, such dysregulation in behavior have also been observed in aged mice subjected to a prolonged exposition to microbiota metabolites and pro-inflammatory cytokines (Caracciolo et al., 2014; Erny et al., 2015; Leung and Thuret, 2015; Manderino et al., 2017). As a further confirmation of the importance of gut microbiota in relation to cognitive decline and impaired behavior, young rodents that have been subjected to FMT from old rodents displayed an aged phenotype represented by impaired motor strength and cognitive behavior (D'Amato et al., 2020; Y. Li et al., 2020; M. L. Wu et al., 2021).

An intriguing question to address regards the possibility that a microbiome belonging to an aged host may aggravate the symptoms in mice with AD background. Therefore, FMT-treated 5XFAD mice were subjected to a battery of behavioral tasks in a fixed-time schedule (e.g., Nesting test in the morning and emergence neophobia in the afternoon) as described in figure 7B. As the stability of the donors' microbiota into the recipient mice was assessed up to 6 weeks after oral inoculation (see Ellekilde et al., 2014), the battery of behavioral tests was planned accordingly within the last 2 weeks. Among the variety of behavioral tasks developed to assess cognitive functions, the radial arm water maze (RAWM), T-maze, Nesting test and emergence Neophobia test are commonly used in AD research and also for evaluating the effect of aging in rodents (Bernaud et al., 2021; Boon and Simpson, 2012; Konsolaki et al., 2016; Leibrock et al., 2013). FMT-treated 5XFAD mice were divided in groups with alternating different types of treatment (young/old), sex (males/females) and genotype (wild type/5XFAD). In addition, aged-matched wild type littermates (from litters not treated with antibiotics but subjected to mock treatment) were used as control mice to prove the applicability of the behavioral tests.

Increased anxiety behavior has been observed in older (18–20 months) as compared to younger (2-6 months) mice and rats and mainly associated to an increase in Firmicutes/Bacteroidetes ratio (Hoffman et al., 2017; Y. Li et al., 2020; Spychala et al., 2018). The anxiety-like behavior was investigated with the emergence neophobia test by measuring the escape latency (in seconds) from a dark container box into a brightfield arena. No differences in escaping time were observed between recipient mice treated with caecum content from either young or old donors. Transgenic mice displayed a general slower escape latency (mean of 10.4 and 11.5 seconds for young and old treatment, respectively) compared to wild type mice (escape latency mean 7.8 seconds). However, no significant differences were detected between untreated wild type mice and FMT-treated groups (wild type versus young $p = 0.238$; wild type versus old $p = 0.087$) (Fig. 15).

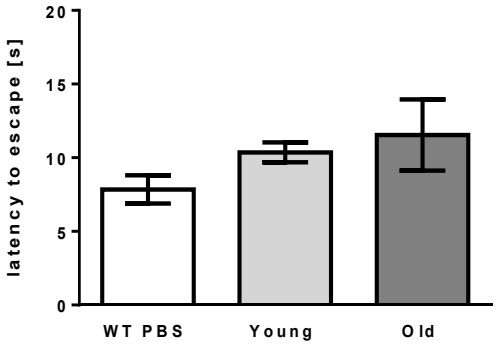



**Figure 15: Analysis of anxiety behavior in Neophobia test.** Time to escape in seconds [s] from a dark container box was measured as an anxiety-reporting parameter. Data are presented as the mean ± SEM ($n$ = 13 for wild type and $n$ = 11 for 5XFAD mice treated with caecum material from either young or old donors). Statistical analysis was performed by using Student's t-test (*, $p < 0.05$). Data are published in Valeri et al. (2021). Abbreviations: Young (treatment with cecal content from young wild type donors), Old (treatment with cecal content from old wild type donors), WT PBS (wild type mice that received mock PBS treatment).

Several aspects of cognitive performances that are normally associated to anatomical and brain changes decline during aging. Among the cognitive functions affected by aging, a decline in spatial orientation and working memory were observed already at 60 years of age and with a more pronounced acceleration around 70 years of age (Barrash, 1994; Beaudet et al., 2015; Verhaeghen et al., 2019). On one hand, spatial orientation memory is defined as the ability of determining and retrieve information from the surroundings maintaining the direction to reach the final destination (Beaudet et al., 2015; Franz and Mallot, 2000; Verhaeghen et al., 2019). On the other hand, working memory is referred to a deductive process that incorporate heurist strategy, comprehension, reasoning, and problem-solving with a small information accessible (Canipe et al., 2021; Cowan, 2014; Tascón et al., 2019). Improvement of working memory together with amelioration in the activation of fronto-parietal and subcortical brain regions, was observed in patients with cirrhosis and cognitive impairment after being treated with rifaximin antibiotic (Ahluwalia et al., 2014). Moreover, consumption of fermented food (enriched with *Lactobacillus brevis BJ20*) resulted in better performance associated to short-time working memory in elderly that can be associated to a protective mechanism against dementia (Reid et al., 2018). Overall, these studies suggest a benefic effect of microbiota associated to aging. Therefore, whether gut microbiota transplant may have an impact on 5XFAD mice cognitive behaviors associated to spatial orientation and working memory, it was investigated by T-maze and RAWM.

Spatial orientation performances in FMT-treated recipient 5XFAD mice were investigated with the T-maze test by measuring the percentage of the right choices (alternation of the left and right arms). No significant differences were detected between different FMT-treated mice ($p$ = 0.4778). However, significant differences were observed between the genetic backgrounds regardless of the type of treatment: wild type mice showed a general higher percentage (75%) in alternating the choice arms compared to 5XFAD mice (50%) (Fig. 16).

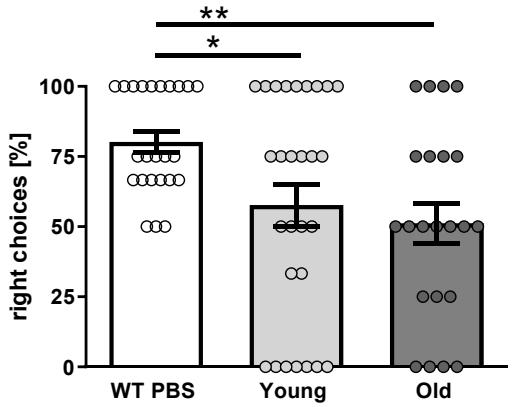



**Figure 16: Spatial orientation in T-maze test.** The percentage of alternating the left with right arm (right choices) in the T-maze was calculated in two consecutive days. Data are presented as the mean ± SEM ($n$ = 13 for PBS-treated wild type (WT PBS), $n$ = 14 for 5XFAD mice treated with caecum material from young (Young) and $n$ = 11 from old (Old) donors)). Statistical analysis was performed by using Student's t-test (*, $p < 0.05$; **, $p < 0.01$). Data are published in Valeri et al. (2021). Abbreviations: Young (treatment with cecal content from young wild type donors), Old (treatment with cecal content from old wild type donors), WT PBS (wild type mice that received mock PBS1X treatment).

Mice learning capability and orientation was also tested in the RAWM. This task is used for fast assessment of AD-related deficits, including working memory (ability to remember/avoid the arms already taken by mice to reach the platforms during each trial) and reference memory (ability to encode spatial information for avoiding arms without escape platforms) (Alamed et al., 2006; Penley et al., 2013; Valeri et al., 2021). In the RAWM task, mice were expected to find either the visible or hidden platform to escape as a way out from the swimming pool. Therefore, to accomplish this task, mice develop a spatial orientation map in the brain using visual cues placed on the four walls surrounding the pool. No differences were also detected among the three groups regarding the RAWM swim speed mean demonstrating the reliability of the task (Fig. 17 A). Learning was assessed by measuring the amount of time elapsed before the mice reach the platform to escape the water (escape latency) and by the numbers of errors committed. Errors in finding the visible platform (trials 1, 3 and 5) were not statistical different between the examined groups indicating a non-compromised visual ability (Fig. 17 B). However, treated 5XFAD mice displayed significant increase in number of errors in finding the hidden platform (trials 2, 4, 6 and 7) in comparison to wild type mice independently from the treatment (Fig. 17 C). In addition, no differences were observed between recipient 5XFAD mice treated with caecum content from either young or old donors ($p$ = 0.6883) (Fig. 17 C). No differences in the latency to escape (in seconds; between trial 7 and 2) to reach the hidden platform was observed between the treated groups ($p$ = 0.6656), while 5XFAD mice displayed a general significant increased latency to escape in comparison to wild type mice independently from the treatment (Fig. 17 D).

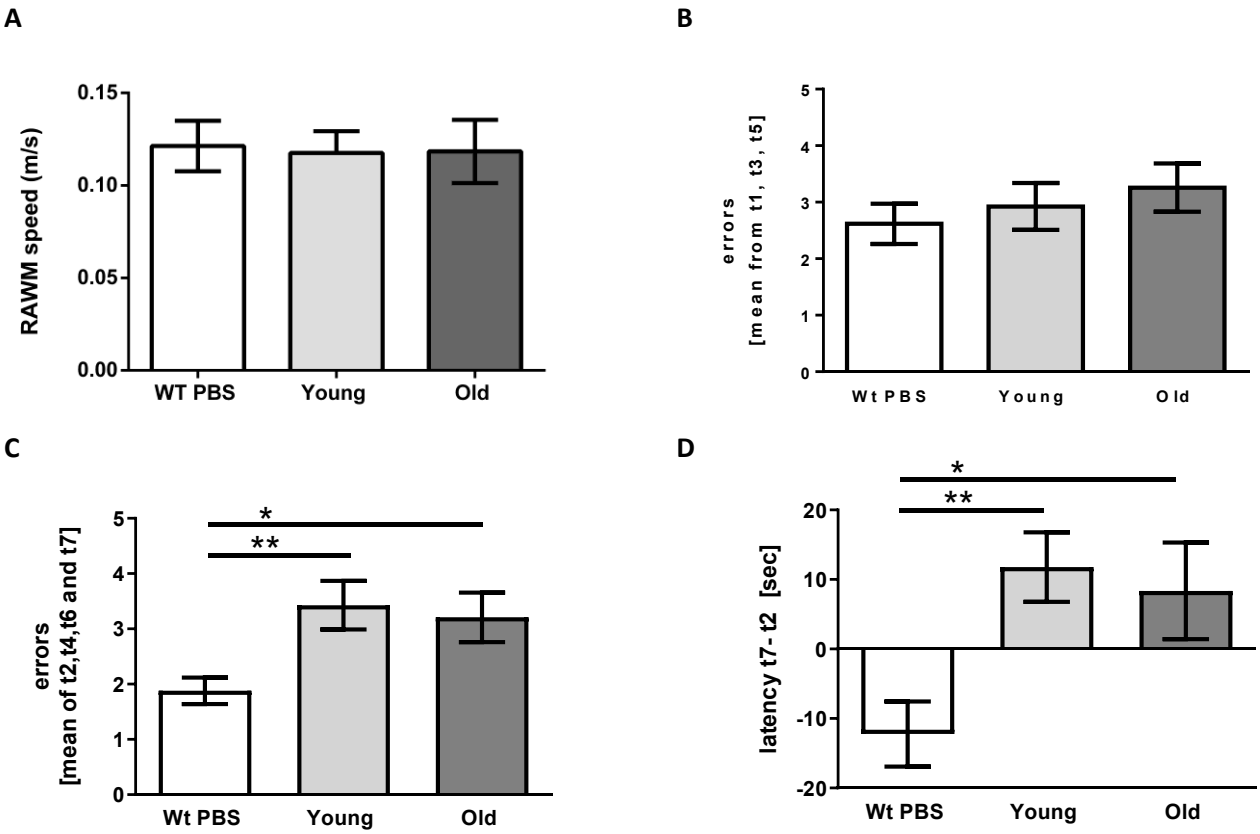



**Figure 17: Spatial learning and orientation in RAWM.** Spatial learning was assessed with respect to the following parameters: errors committed (A, B) and the escape latency to find the platform (C). (A) Visible and hidden platforms were alternated up to the 5$^{th}$ trial, while the last two trials (6$^{th}$ and 7$^{th}$) were conducted only with the hidden platform. Errors committed by the mice with visible platform were counted within three trials (t1, t3, and t5) to exclude visual deficits. (B) Errors committed by the mice with the hidden platform (t2, t4, t6, t7) only. (C) Latency to escape was calculated taking into account the differences for finding the hidden platform between t7 and t2. Data are presented as the mean ± SEM (*n* = 13 for wild type, *n* = 14 for 5XFAD mice treated with caecum material from young (Young) and n = 11 for mice treated with old (Old) donor material). (D) Mice speed (m/s) was monitored along the duration of the test by video camera system (ELP, Shenzen, Guangdong, China) and analyzed with the Anymaze software (version 6.1; Stoelting Europe, Dublin, Ireland). Statistical analysis was performed by using Student's t-test (*, $p < 0.05$; **, $p < 0.01$). Data are published in Valeri et al. (2021). Abbreviations: Young (treatment with cecal content from young wild type donors), Old (treatment with cecal content from old wild type donors), WT PBS (wild type mice that received mock PBS1X treatment), t 1,2,3,4,5,6,7 (trials).

In summary, behavioral examinations of transplanted 5XFAD mice did not show significant differences between the young and old group regardless of the task used. In particular, it is well known that transgenic 5XFAD mice start to develop their first behavioral deficit related to AD pathology around the fourth months of age (Devi and Ohno, 2010; Locci et al., 2021; Oakley et al., 2006; Schneider et al., 1999; chapter 1.5). Therefore, lack of differences in most of the used behavioral tasks, due to the received treatment (cecal content from young or old wild type donors), may be attributed to a precocious age of investigation in treated 5XFAD mice (final age of 10 weeks). Future studies on the early onset (above 4 or 5 months of age) in 5XFAD mice are needed. To date, no other studies showed the effect of microbiota transplantation in relation to aging specifically in 5XFAD transgenic mouse model. So far, the unique report addressing the effect of fecal transplant from healthy wild type into AD mice, showed improvements in behavioral tasks (e.g., Morris water maze and object recognition tests) in APPswe/PS1ΔE9 transgenic mice aged 6 months (Sun et al., 2019). However, neither the age of the donors was described nor a direct comparison between a "young" and "aged" microbiota on recipient mice was investigated (Sun et al., 2019). In this study, although no significant changes between mice treated with caecum content from young and old donors were detected, promising results were shown in some of the behavioral tasks described. In the neophobia test, no differences in

anxiety-dependent behavior, measured with latency to leave the box, were detected either between the young and old group of treatment and either between transgenic or wild type mice. Contrarily, in both the spatial orientation-based tasks (i.e., the T-Maze and RAWM tests), behavioral deficit for 5XFAD mice as compared to wild type littermates were already observed at this early stage. In particular, the RAWM is a hybrid of the Morris water maze and a radial arm maze that permit a fast assessment of learning deficits associated to errors in the localization of the goal arm and to delay to reach the platform using water immersion as motivation factor (Alamed et al., 2006). T-maze test was used to evaluate the working memory (i.e., the mice behavioral response in each trial change in relation to previous attempts) represented by the mice natural tendency to spontaneously alternate the two goal arms (Deacon and Rawlins, 2006). In addition, T-maze was preferred to the mainly used Y-maze for its rigidity in making distinct choices between the left and the right goal arms. Probably the choice of the T-maze rather than the Y-maze allowed a more efficient early detection of behavioral learning deficits in 5XFAD mice. Nevertheless, the only changes observed were attributable to genetic background, while no differences were observed between the two groups of treatments.

## 4.5 Gut morphology analysis in FMT-treated and mock-treated 5XFAD mice

Intestinal morphology exerts a crucial function in the absorption and metabolism processes (Kuzmuk et al., 2005). Moreover, changes in the gut microbiota composition during aging may affect the intestine architectural structure predisposing the host to the development of disorders (Kuzmuk et al., 2005). Functional decline in the regenerative potential of crypts has been observed in the small intestine of aged mice pointing out to gut dysbiosis (Martin et al., 1998a, 1998b). To date, only one investigation showed increased crypts depth and villi length in aged (18-22 months of age) C57BL/6 mice compared to younger (2-4 months of age) ones (Nalapareddy et al., 2017), while a total lack of knowledge is concerning the transgenic 5XFAD mouse model. Moreover, an increase in submucosa and the muscularis thickness were also observed in duodenal samples of 12-month-old NMRI/Bom mice in comparison to younger 3-month-old animals (El-Salhy et al., 1999). Intestine crypts represent a highly proliferative amplifying zone for the villi development (Barker et al., 2008). In addition, villi and crypts length have often been used as an indicator of intestine health (Wiersema et al., 2021) as reduced villus height and crypt depth may alter the nutrient absorption (Kuzmuk et al., 2005). Enhanced permeability in addition to altered sugar and peptide absorption in small intestine was associated to reduction in villus length and crypt depth upon induction of inflammation in experimental models (see review Peuhkuri et al., 2010). Changes in the gut morphology may impact some gut functions, such as the transit time for expelling feces from the gut in mice (Stoye et al., 2020). Therefore, specific architectural structure parameters in the duodenal region of the intestine, including villus length, crypt depth, submucosa and muscularis thickness were analyzed in relation to genotype of the mice (wild type versus 5XFAD mice) and in relation to transplant of aged microbiota in 5XFAD mice. A comparison between age-matched wild type and 5XFAD mice gut morphology was published in Nguyen et al. (2021), while the effect of FMT on the host gut morphology in 5XFAD mice was investigated in Valeri et al. (2021). Immunohistochemistry of intestine samples were performed by [redacted]; Core facility Immunohistochemistry of the University Medical Center Mainz (collection and preparation of intestine tissues were described in chapters 2.2.8 and 2.2.10.1, while immunostaining procedure was reported in Nguyen et al. (2021) and in Valeri et al. (2021)). Firstly, differences in gut morphology parameters were analyzed between age-matched transgenic and wild type mice treated with a mock solution to reproduce the same stress of mice subjected to fecal transplant (Fig. 18 A). No differences were observed in the mentioned morphological structures due to the genotype aside from a significantly increased muscular layer height in transgenic mice (Fig. 18 B). Such observed increase in the muscular layer height of 5XFAD mice might be a consequence of the inflammation process, a common feature in AD and in other diseases, such as Crohn's disease (Kinney et al., 2018; Stenke et al., 2017). Changes in the intestine architecture structure according to aging have been described in several studies both in humans and rodents (reviewed by Drozdowski and Thomson, 2006; Thomson, 2009). However, divergent observations have been made. In detail, rats and rabbits showed reduced villus height and surface area of the proximal small intestine with age (Höhn et al., 1978; Holt et al., 1984; Keelan et al., 1985), while no changes in the previously described gut structures were

found in humans (Corazza et al., 1986; Lipski et al., 1992; Webster and Leeming, 1975). Differently, Martin et al. (1998a) reported an increase in villus height but a decrease in the number of villi and crypts with mouse age.

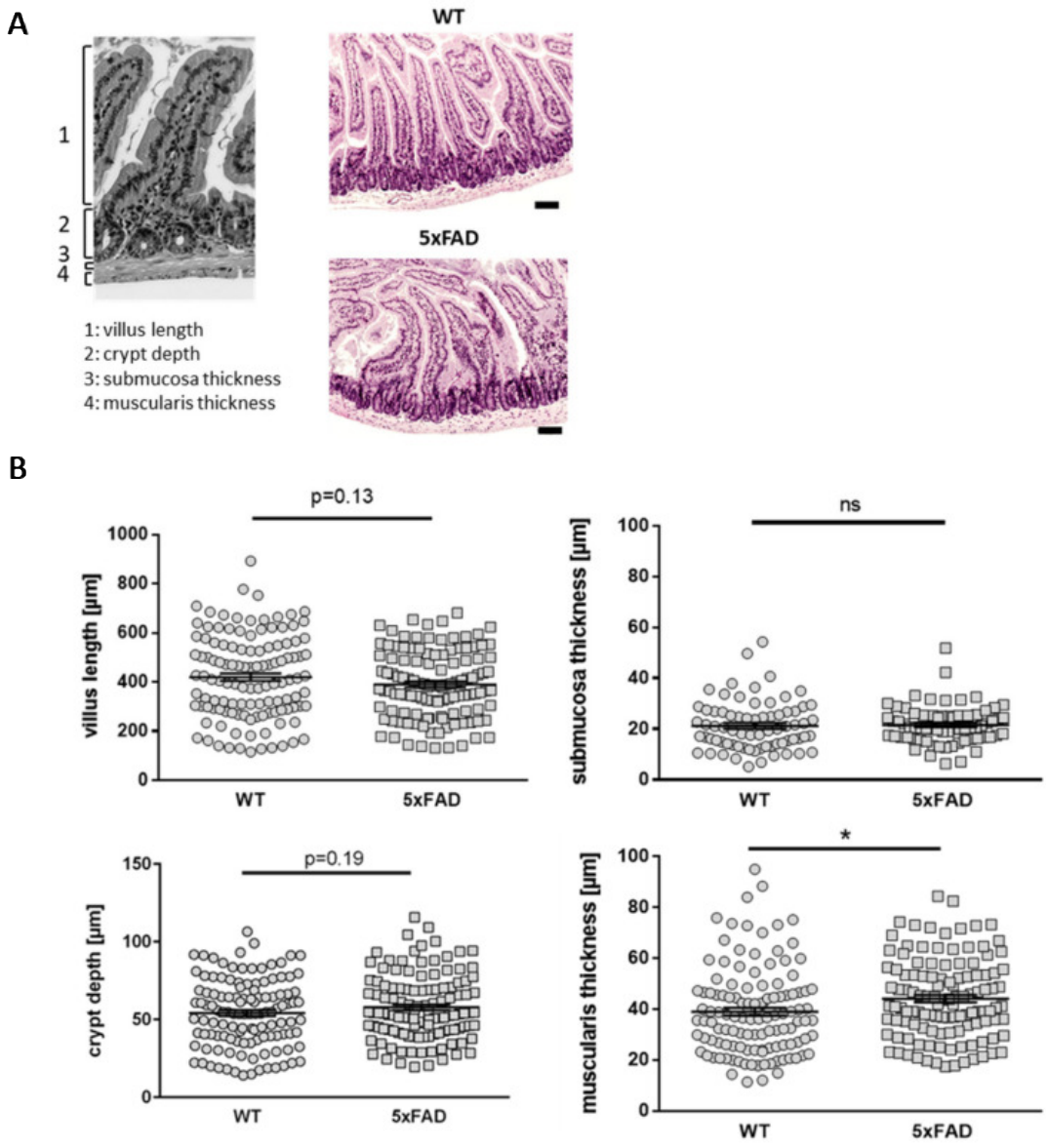



**Figure 18: Analysis of duodenal morphological structures in mock-treated wild type and 5XFAD mice.** (A) Representative hematoxylin and eosin (HE) staining of intestinal duodenum structures (i.e., villus length, crypt depth, submucosa and muscularis thickness) were assessed in mock-treated (treated with PBS1X) 5XFAD and wild type mice. (B) Comparison of duodenal structures due to genetic background (three slides per mouse, *n* = 10 animals per group, *n* = 5 females). Four villi and neighboring structures were analyzed per slice by using a clockwise orientation to avoid selection bias (structures were measured at 3, 6, 9, and 12 h when seeing the gut cross-section as a clock). Immunohistochemistry of intestine samples was performed by [redacted]; Core facility Immunohistochemistry of the University Medical Center Mainz; method described in Valeri et al. (2021). Data are presented as the mean ± SEM. Statistical analysis was performed by using Student's t-test (*, $p < 0.05$). Exemplary gut sections and data were published in Nguyen et al. (2021). Abbreviations: 5XFAD (Alzheimer's disease mouse model), WT (wild type), HE (hematoxylin and eosin), ns (not statistically significant).

Whether aging and gut microbiota are associated or not to intestinal morphological changes is still an open question. Therefore, in this chapter, the effect of aged microbiota transplant was evaluated in 5XFAD mice after 6 weeks from the inoculation (Fig. 19 A). Although no differences in the crypt depth were observed in transgenic animals after being exposed to the two treatments, the villus length, the submucosal and muscularis thickness were significantly increased in 5XFAD mice receiving cecal material from old donors (Fig. 19 B, C, D and E).

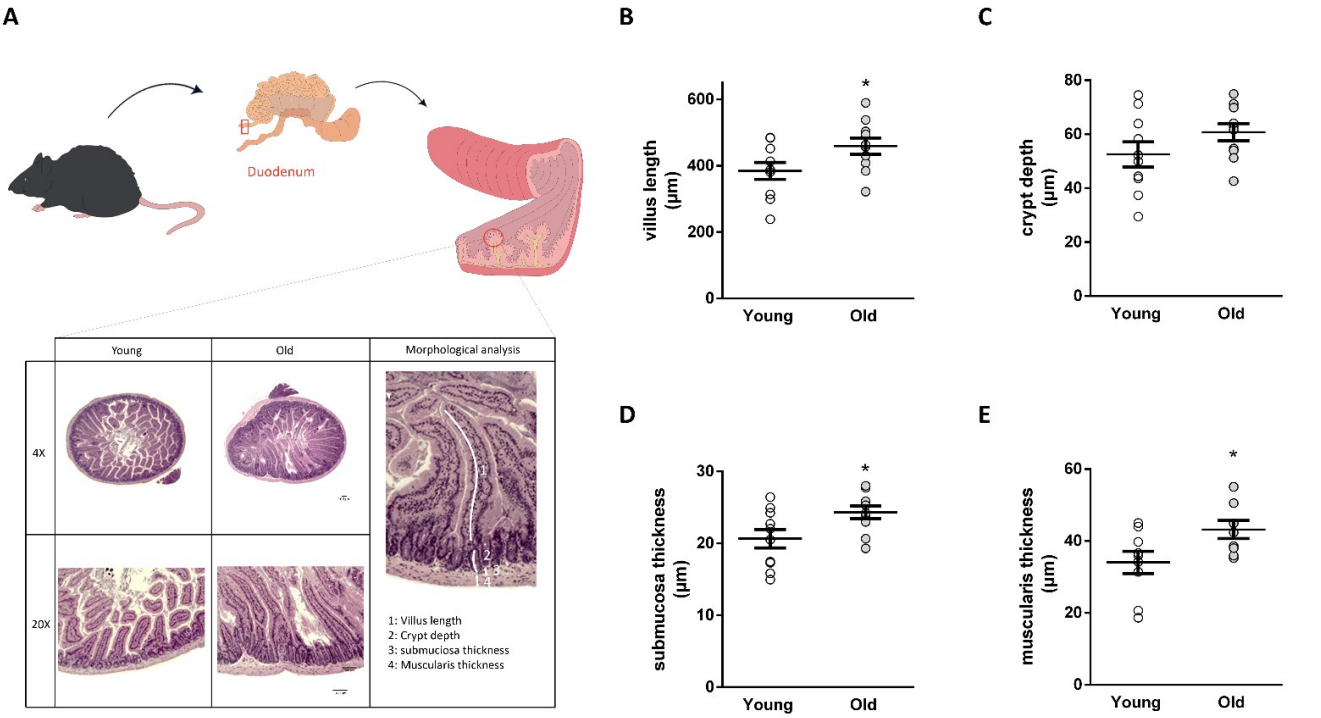



**Figure 19: Analysis of duodenal morphological structures in FMT-treated recipient 5XFAD mice.** (A) Representative hematoxylin and eosin (HE) staining of intestinal duodenum structures (i.e., villus length, crypt depth, submucosa and muscularis thickness) were assessed in FMT-treated 5XFAD mice. Comparison of duodenal structures between young and old treated groups of 5XFAD mice (three slides per mouse, *n* = 10 animals per group, *n* = 5 females): villus length (B), crypt depth (C), submucosa (D) and muscularis thickness (E). Four villi and neighboring structures were analyzed per slice by using a clockwise orientation to avoid selection bias (structures were measured at 3, 6, 9, and 12 h when seeing the gut cross-section as a clock). Immunohistochemistry of intestine samples was performed by [redacted]; Core facility Immunohistochemistry of the University Medical Center Mainz; method described in Valeri et al. (2021). Data are presented as the mean ± SEM. Statistical analysis was performed by using Student's t-test (*, $p < 0.05$). Exemplary gut sections and data were published in Valeri et al. (2021). Abbreviations: Young (treatment with cecal content from young wild type donors), Old (treatment with cecal content from old wild type donors), HE (hematoxylin and eosin).

In summary, mock-treated aged-matched wild type and 5XFAD mice present significant difference only in one of the intestine's anatomic structures investigated, the muscularis thickness (Fig. 18 B). Moreover, the transplant of microbiota in recipient transgenic 5XFAD mice was able to, at least partially, recreate the physiologic condition described in the intestine during normal aging. The observed differences in the muscularis thickness due to the genetic background were accentuated also after the microbiota transplant in the old group. Therefore, in the here presented study, it may be assumed that the regenerative stem cells and other gut functions that were reported to be negatively affected by aging, may also be compromised after administration of microbiota derived from aged mice. It is also important to take into consideration that duodenum presents a lower microbial density ($10^3$ bacteria/gram) when compared to other intestinal regions, such as ileum ($10^7$ bacteria/gram) and colon ($10^{12}$ bacteria/gram) (see review Dieterich et al., 2018).

Since modifications in the morphology were already visible in duodenum samples due to FMT from old wild type donors, it is plausible to assume that ileum and colon regions, characterized by higher microbial density, may potentially show even more evident changes in such gut structures. In addition, elongation in some of the here presented gut structures may also be associated with absorptive dysfunctions due to the aging effect as also described elsewhere (Holt, 2001; Riordan et al., 1997).

## 4.6 Physiological parameters associated to aging in 5XFAD FMT-treated recipient mice

A common condition in elders is represented by increased low-grade inflammation (see chapter 1.9). Alteration of certain bacteria in the elderly (see Table 2) is associated with gut dysbiosis and with impaired intestinal epithelial integrity. Lack of epithelial integrity is also considered as one of the main causes for the increase in gut permeability ("leaky gut") and systemic endotoxemia (Ragonnaud and Biragyn, 2021). The endotoxin LPS, a component of the bacterial cell wall of Gram-negative strains, is considered a strong trigger for the inflammatory pathway (Kell and Pretorius, 2015). In addition, LPS-binding protein (LBP) is a serum soluble glycoprotein (60 KDa) that is mainly synthetized in the liver by hepatocytes (Grube et al., 1994; Ramadori et al., 1990; Schumann et al., 1996) and in the intestine by epithelial cells (Vreugdenhil et al., 1999), and involved in innate immune response. It exerts a crucial function in the early detection of LPS facilitating the binding of LPS to CD14 in CD14+ cells and thereafter, activating transduction pathways involved in the release of pro-inflammatory mediators in the serum (Guha and Mackman, 2001; Wright et al., 1990). Increased LBP levels were also associated to acute inflammatory responses in sepsis (K. F. Chen et al., 2016) and in fat and carbohydrates rich diets (Ghanim et al., 2009).
Analysis of plasma samples in C57BL/6J mice revealed higher levels of the LBP in aged 28-months-old compared to younger 6-months-old male mice (van der Lugt et al., 2018). Based on this study, an intriguing question regards the possibility that the transplant of an aged microbiota may induce an increase in the LBP levels also in 5XFAD recipient mice and with this, inducing an increase in the gut permeability. Therefore, analysis of LBP concentration in 5XFAD mice serum samples 6 weeks after the transplant was carried out by [redacted] (Department of Psychiatry and Psychotherapy, Johannes Gutenberg-University, Mainz; description of methods and result in Valeri et al. (2021)). Interestingly, a significant increase (around 25%) of LBP concentration was observed in 5XFAD mice treated with cecal content from old donors in comparison to those treated with material from young wild type donors (Fig. 20).

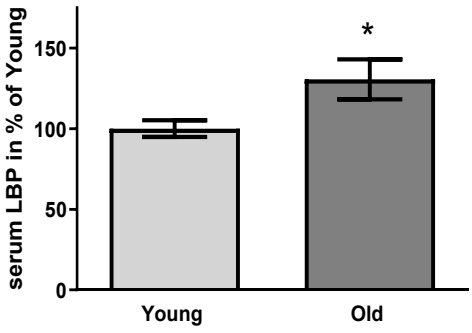



**Figure 20: Analysis of LBP concentration in serum samples of treated recipient 5XFAD mice.** LBP analysis was collected from serum samples and analyzed by ELISA (*n* = 11 per group: young and old FMT-treated 5XFAD mice). Data are presented as the mean ± SEM. Statistical analysis was performed by using Student's t-test (*, $p < 0.05$). Analysis performed by the [redacted] (Department of Psychiatry and Psychotherapy, Johannes Gutenberg-University, Mainz; description of methods and result in Valeri et al. (2021)). Abbreviations: Young (treatment with cecal content from young wild type donors), Old (treatment with cecal content from old wild type donors), LBP (LPS binding protein).

However, LBP levels were only addressed in serum samples, while brain parenchyma samples were not investigated. Since the effect of LBP was limited only to 25%, it may be assumed that aged donors' transplant exerts its function only in the periphery of recipient mice without reaching/affecting the brain.
Alteration in metabolic pathways is often associated to decreased energy consumption and increased body weigh that may cause several complications associated to inflammation state, such as hypertension, cardiovascular disease and diabetes, in the elderly (Khanam et al., 2011; McTigue et al., 2006; Villareal et al., 2005). As previously introduced (chapter 1.7), gut microbiota exerts a fundamental role in the control of the host energy balance through the modulation of several metabolic pathways. Moreover, the gut microbiota structure is also shaped by both age and blood glucose levels as demonstrated by more abundant *Lactobacillus* and *Bifidobacterium* at genus levels in participants belonging to high blood glucose concentration group (Enqi et al., 2019). Alteration in blood glucose levels is responsible for microbial dysbiosis and, for the changes in the production of associated metabolites, such as the SCFAs, which in turn, are involved in the regulation of immune system functions. In particular, decreased levels of SCFAs due to changes in blood sugar levels have been associated to increased concentration of LPS and to low-grade inflammatory status (Enqi et al., 2019). Therefore, since 5XFAD-treated mice with cecal content from old wild type donors showed increased levels of LBP (Fig. 20), blood sugar levels were evaluated in the assigned groups (Fig. 21). No differences were observed between the two treatments suggesting the existence of a different regulation mechanism between mice and humans. To not interfere with the behavioral outcomes, mice were not kept at a particular diet regime or either be fasted the days before their dissection. However, mice were daily monitored and no differences in their body weight, that may affect the described evaluation, were observed in the treated groups (Table 3). In addition, it has been reported that the use of antibiotics affects the blood sugar and serum glucagon levels in 5XFAD mice (Dos Santos Guilherme et al., 2021). Since mice were pre-treated with antibiotics, before being inoculated with cecal content provided by healthy donors, it is possible to speculate that such treatment may interfere with the current evaluation of the blood sugar concentration in the FMT-treated groups.

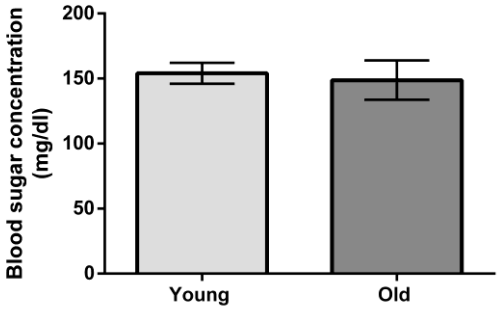



**Figure 21: Sugar levels in blood samples of treated recipient 5XFAD mice**. Samples from truncal blood were collected from mice during the dissection and blood sugar levels quantified (*n* = 14 for young and *n* = 12 for old FMT-treated 5XFAD mice). Data are presented as the mean ± SEM (unpublished result). Statistical analysis was performed by using Student's t-test (*, $p < 0.05$). Abbreviations: Young (treatment with cecal content from young wild type donors), Old (treatment with cecal content from old wild type donors).

Increased BBB permeability in the elderly may facilitate the infiltration of immune cells in the brain and thereby accelerate the neuroinflammation process in AD patients (review Seo and Holtzman, 2020). In addition, both LPS and gut leakage may contribute to dysbiosis-related AD pathogenesis amplifying the Aβ deposition in the brain (Seo and Holtzman, 2020). Aside low-grade inflammation, another physiological feature associated to aging is represented by increased amount of visceral adipose tissue both in humans and rodents (Bartlett et al., 2012; Houtkooper et al., 2011; Hunter et al., 2010; Matsuzawa et al., 1995; Wu et al., 2007). Moreover, recruitment of thermogenic tissues, such as the brown and white adipose ones, have

been described to be controlled by gut microbes acting on the regulation of blood glucose concentration (Chevalier et al., 2015; Li et al., 2019; Somm et al., 2017; Worthmann et al., 2017).
Abdominal fat, an anatomic region widely studied in rodents for its metabolic activity in adipose tissue, was collected and analyzed between 5XFAD mice treated with cecal content from young and old wild type donors. Analysis of abdominal fat (mg/g of body weight) revealed no significant differences between the two groups of mice after 6 weeks from the treatment. However, a trend to increase was observed in recipient mice treated with cecal material from old wild type mice ($p$ = 0.06) (Fig. 22).

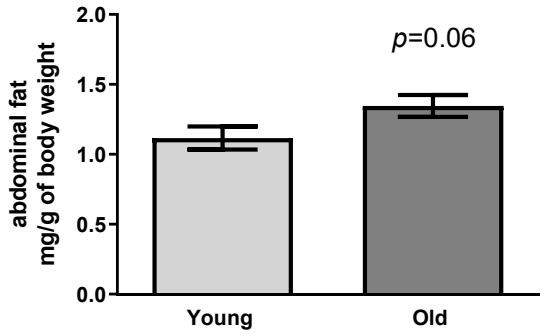



**Figure 22: Analysis of abdominal fat in treated recipient 5XFAD mice.** Abdominal fat was collected and weighed after animals' dissection ($n$ = 11 per group: young and old FMT-treated 5XFAD mice). Data are presented as the mean ± SEM in percentage of 5XFAD-treated young ("Young") mice. Statistical analysis was performed by using Student's t-test (*, $p$ < 0.05). Data are published in Valeri et al. (2021). Abbreviations: Young (treatment with cecal content from young wild type donors), Old (treatment with cecal content from old wild type donors).

Although levels of blood sugar were not different between the two groups (Fig. 21), transplantation of cecal content from old wild type donors was able to cause a mild increase in the abdominal fat concentration in 5XFAD mice (Fig. 22). Probably, the lack of statistical significance in the described results may be limited by the timeline adopted in the current investigation. Indeed, it is possible to assume that prolongation of this study (more than six weeks after FMT) may generate higher statistical power.
Taken together, all the analyzed features in the old groups, such as increased serum LBP levels and abdominal fat mass (Fig. 20 and 22), but also increased length in gut structures (Fig. 19), share a common profile attributed to the physiologic aging process in humans and animals. It may be assumed that cecal content transplant from old mice in young (P29 at the day of FMT) recipient 5XFAD mice may induce aging-associated changes that, not only directly influence the gut, but also may have wider systemic consequences. In particular, aging is often accompanied by alteration in body temperature with high variability in humans (Balmain et al., 2018; Waalen and Buxbaum, 2011). In the vast majority of reported cases/studies, humans' elderly and aged mice showed reduced body temperature compared to younger individuals (Güneş and Zaybak, 2008; Howell, 1948; Salvosa et al., 1971). It has been suggested that the drop in body temperature observed in elders may be caused by their inability to regulate their body temperature in equal measure as young adults because of alterations in their responses to changes in body temperature (Güneş and Zaybak, 2008). Moreover, microbiome composition and other gut-related functions (e.g., digestion process) are dependent from the host temperature (Hylander and Repasky, 2019). In addition, adipose tissue exerts its function not only in storing the energy, but also in the production of heat (Song et al., 2013). Indeed, people suffering of obesity present a thicker layer of subcutaneous adipose tissue that can be associated with their higher body temperature (Bastardot et al., 2019). Therefore, the body temperature of the mice was evaluated in FMT-treated 5XFAD mice. Body temperature was always measured at the same hour (around 9:00 AM) of the morning to avoid errors due to temperature circadian fluctuation as described elsewhere (Hankenson et al., 2018). Interestingly, after 6 weeks from FMT, mice treated with cecal material from old

donors showed a significant reduction of 1% of the body temperature compared to the ones treated with material from young donors (Fig. 23).

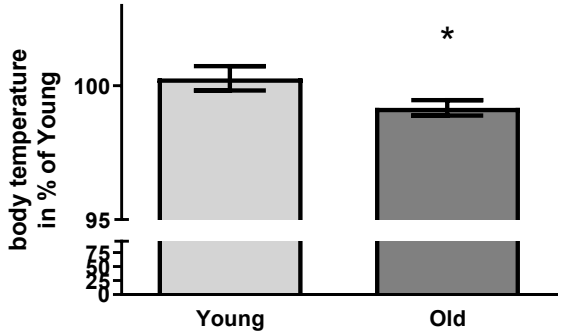



**Figure 23: Analysis of body temperature in FMT-treated recipient 5XFAD mice**. Body temperature was assessed directly before sacrifice (around 9:00 AM) using an infrared thermometer on the genital area. Temperature was taken into account only in the cases of not defecation or urination during measurement. Data are presented as the mean ± SEM in percentage of 5XFAD-treated young ("Young") mice (5XFAD mice treated with caecum content from young ($n$ = 8) and old ($n$ = 10) wild type donors). Statistical analysis was performed by using Student's t-test (*, $p < 0.05$). Data published in Valeri et al. (2021). Abbreviations: Young (treatment with cecal content from young wild type donors), Old (treatment with cecal content from old wild type donors).

It is possible to assume that transplant of cecal microbiota from healthy old wild type donors into recipient transgenic 5XFAD mice may induce changes in body temperature that are associated to aging. Several parameters need to be taken into account for the assessment of body temperature, including the already cited daily fluctuation but also, the different handling stress caused to animals when they are restrained for temperature assessment and the methodology used. For the methodology, sensors inserted under the loose skin of the pelt of mice were able to confirm the observed drop in body temperature associated with aging (Gonzales and Rikke, 2010). Other common methods consist in using rectal probes for temperature readings, which requires the use of anesthetic in order to insert the probe without causing harm to animals (Kawakami et al., 2018). However, in this study, the infrared thermometer allowed the detection of the body surface temperature without subjecting the mice to undue stress (Kawakami et al., 2018). In addition, reliability of infrared thermometer was already tested in comparison to the rectal probes (Kawakami et al., 2018).
Among the behavioral mechanisms used by animals for conserving the body temperature, "huddling" and "nest building" tests are considered as valuable indicators of mice health and well-being (Gaskill et al., 2013; X. Y. Zhang et al., 2018). Huddling is a behavioral mechanism used by animals for conserving the body temperature that is also able to alter the microbiota composition in rodents (X. Y. Zhang et al., 2018). In addition, X. Y. Zhang et al. (2018) suggested that the temperature variation and energy consumption due to huddling are associated with the shaping of the cecal microbiota in voles' (X. Y. Zhang et al., 2018). Nesting is a task based on a passive learning behavior that rely on two brain regions, such as hippocampus (Deacon, 2012; Deacon and Rawlins, 2006) and prefrontal cortex (Kolb and Whishaw, 1985). In detail, hippocampal neurons called "nest cells" were deputed to the nest building function (Lin et al., 2007) as demonstrated by poor nests built in mice and rats with hippocampal lesions (Deacon et al., 2002; Kim, 1960). In the presented study, nest building ability was evaluated after 6 weeks from FMT treatment in 5XFAD mice following a rigid planned time schedule (described in Fig. 7B). Interestingly, transgenic mice treated with cecal content from old wild type mice donors displayed, even if not statistically significant, higher score and reduced amount of unused material when compared to the ones treated with cecal content from young wild type individuals (Fig. 24 A and B).

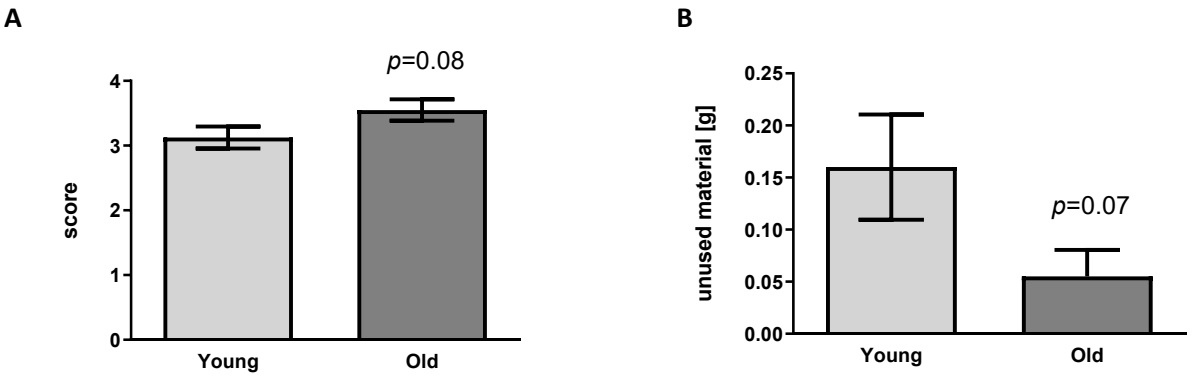



**Figure 24: Nest building behavior in FMT-treated recipient 5XFAD mice.** (A) Nesting score and (B) unused material in 5XFAD mice after 6 weeks from the FMT treatment. Data are presented as the mean ± SEM ($n$ = 20 per group, $n$ = 5 females). Statistical analysis was performed by using Student's t-test (*, $p < 0.05$). Data are published in Valeri et al. (2021). Abbreviations: Young (treatment with cecal content from young wild type donors), Old (treatment with cecal content from old wild type donors).

It appears plausible, that the observed reduction in body temperature (Fig. 23) may affect the tendency to build better nest structures with more material integrated in 5XFAD mice treated with cecal content from old donors. Therefore, it is possible to speculate that mice, that received cecal material from old donors, build better nest with more integrated material to balance the loss of body temperature due to the gut microbiota transplant.
In summary, this study showed that transplant of cecal microbiota belonging to aged mice into young recipient 5XFAD mice reproduced some physiological changes occurring in aged animals and humans and that such changes were already visible after 6 weeks from transplantation.

## 4.7 Effect of FMT on brain hallmarks in 5XFAD mice

LPS and gut leakage may facilitate the infiltration of immune cells in the brain and with this, contributing to acceleration of dysbiosis associated to AD pathogenesis, such as the amplification of Aβ deposition in the brain (Seo and Holtzman, 2020). As previously reported (chapter 4.6), FMT-treated 5XFAD mice with caecum content from old donors displayed higher LBP concentration and alteration of other aging-related physiological parameters (Figs. 20-23). In addition, molecular changes due to the pathology have already been described in human patients before the onset of clinical symptoms (Jack et al., 2013). Previous studies conducted in APPswe/PS1ΔE9 mice revealed amelioration of AD symptomatology after FMT treatment (Dodiya et al., 2019; Sun et al., 2019). Sun et al. (2019) reported an improvement in cognitive deficits together with decrease of Aβ in APPswe/PS1ΔE9 mice (aged 6 months) after being subjected to FMT treatment. Such improvements were also accompanied by increased synaptic plasticity (i.e., increased PSD-95 and synapsin I level) (Sun et al., 2019). Similarly, a restoration of microbiome diversity and profile together with, a partial restoration of Aβ plaques and microglial morphology, were observed in antibiotic-treated APPswe/PS1ΔE9 male mice after being exposed to fecal transplant from age-matched APPswe/PS1ΔE9 male donor mice (Dodiya et al., 2019). Therefore, such studies revealed that fecal microbiota is involved in AD pathology and presumably acting responsible for the Aβ deposition and microglial physiology in AD mouse model (Dodiya et al., 2019).
Whether FMT from healthy donors may induce aggravation of AD hallmarks specifically in the brain of recipient 5XFAD mice in relation to aging has not been addressed so far. Therefore, in this study, brain histological quantitative analysis was conducted in 5XFAD-treated mice (immunohistochemistry of brain samples was performed by [redacted]; Core facility Immunohistochemistry of the University Medical Center Mainz; collection and preparation of brains for immunohistochemistry staining was described in

chapter 2.2.8 and 2.2.10.2.1, while immunostaining procedure was reported in Valeri et al. (2021)). The representative brain regions of cortex, prefrontal cortex, hippocampus (i.e., dentate gyrus and subiculum) were chosen as target areas for their involvement in AD (brain areas affected by AD are described in chapter 1.4). No differences in Aβ-peptide containing deposits were detected in all target brain areas stained with the antibody 6E10 independently from the treatment (Fig. 25 A, B and C). Such lack of differences between the two treated groups in the selected brain areas may be explained by the use of less selective antibody for Aβ. Indeed, 6E10 antibody allowed the detection of all abnormally processed forms derived from APP (reactive to amino acids 1-16 Aβ) as well as for the full-length APP itself. However, quantitative analysis with Thioflavin-T (ThT), a fluorescent dye commonly used to detect amyloid plaques, revealed higher signal intensity in 5XFAD recipient mice treated with cecal content from old wild type mice in the prefrontal cortex and dentate gyrus areas as compared to the young-treated ones (Fig. 26).

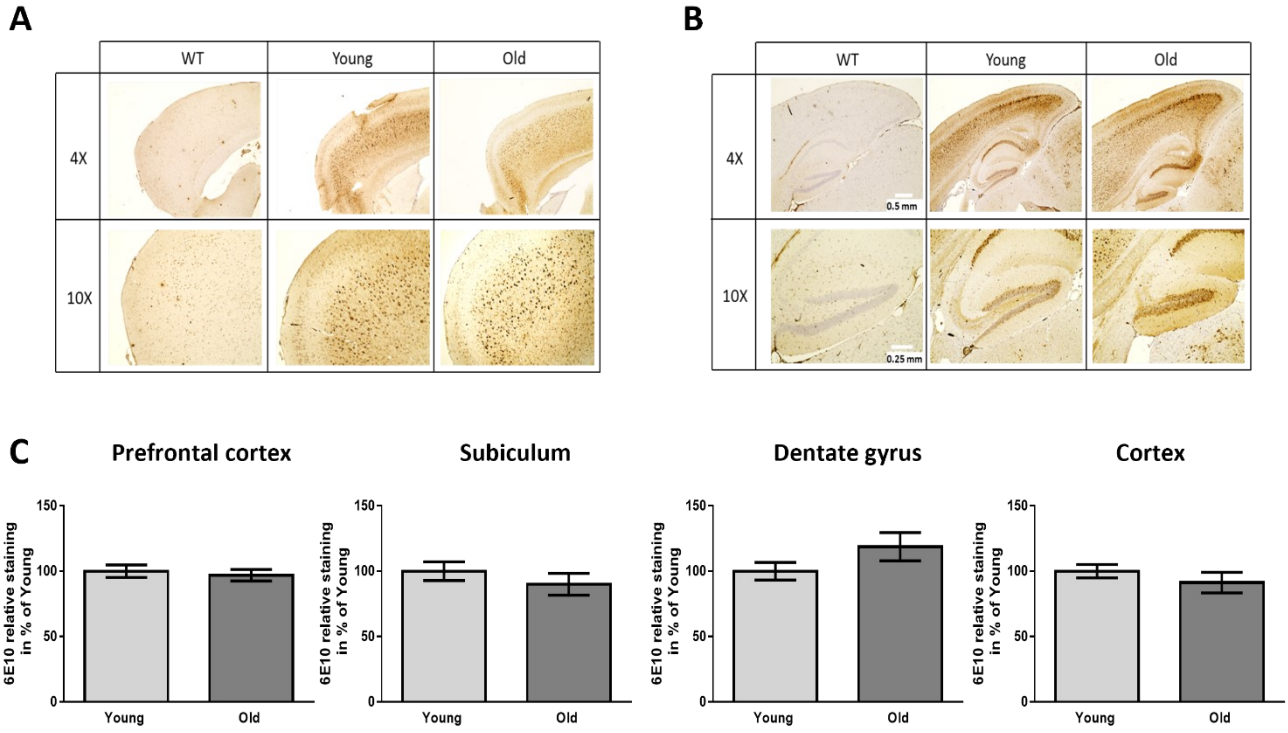



**Figure 25: Staining of Aβ-containing deposits in selected brain regions of FMT-treated recipient 5XFAD mice.** Exemplary representation of APP-derived cleavage products staining (antibody 6E10) in the prefrontal cortex (A) and in the dentate gyrus, subiculum and cortex regions (B) of 5XFAD mice treated with cecal content from young and old wild type mice compared to wild type (WT) mice. Scale bars represent 0.5 and 0.25 mm (4 x and 10 x magnification). C) Quantitative analysis of antibody 6E10 intensity using defined area-sizes as described elsewhere (Reinhardt et al., 2018). Data are presented as the mean in percentage of young ("Young") group ± SEM. Two slides per mouse were analyzed ($n$ = 9 for transgenic mice treated with cecal material from young and $n$ = 10 for mice treated with old donor material). Statistical analysis was performed by using Student's t-test (*, $p < 0.05$). Immunohistochemistry of brain samples was performed by [redacted]; Core facility Immunohistochemistry of the University Medical Center Mainz; method described in Valeri et al. (2021). Data are published in Valeri et al. (2021). Abbreviations: Young (treatment with cecal content from young wild type donors), Old (treatment with cecal content from old wild type donors), WT (wild type).

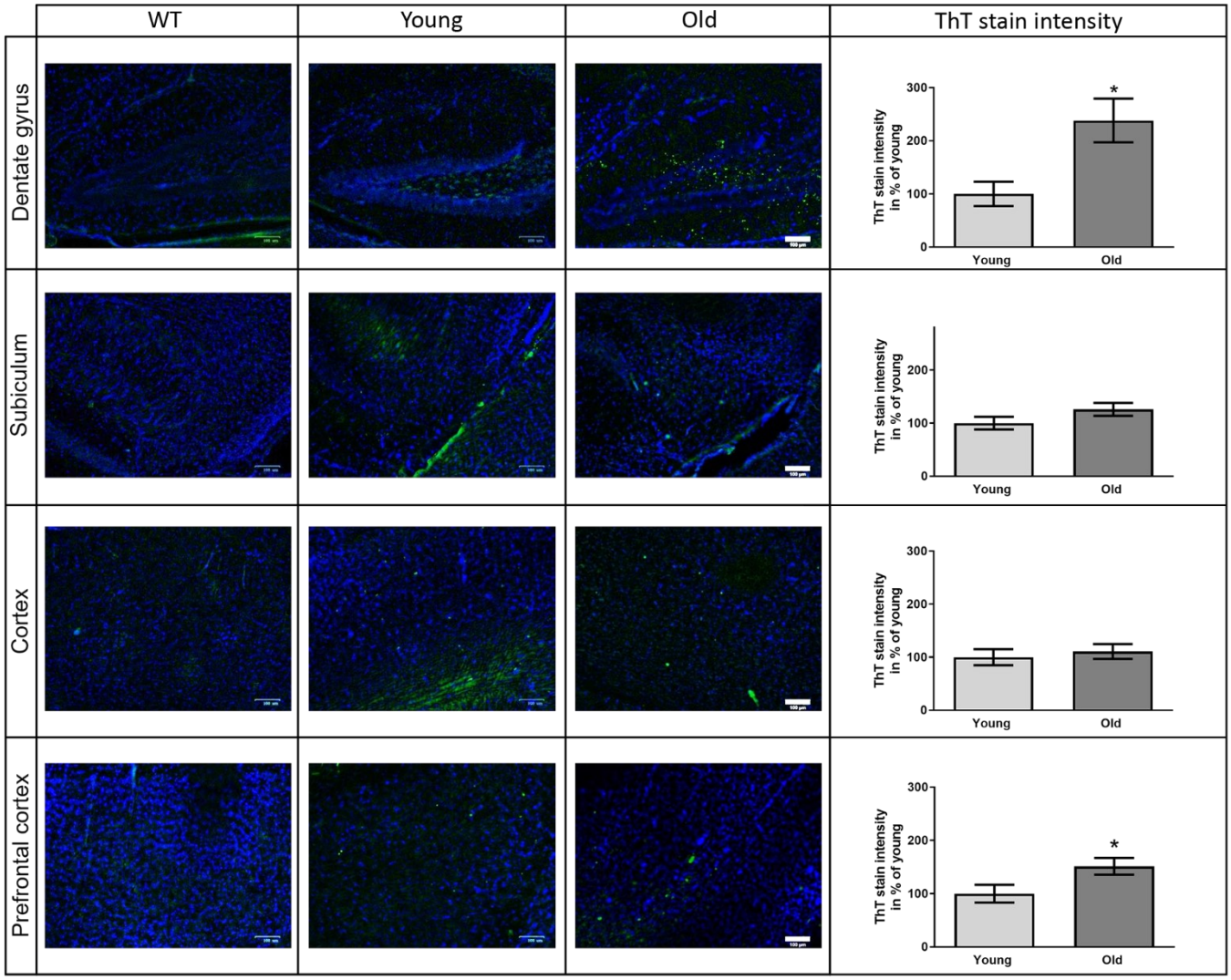



**Figure 26: Amyloid plaques staining in brain areas of FMT-treated recipient 5XFAD mice.** (Left) Representative stained brain areas (i.e., dentate gyrus, subiculum, cortex and prefrontal cortex) with Thioflavin-T (green) for labelling amyloid plaques and DAPI (blue) for detecting the neuronal nuclei double-stranded DNA in untreated wild type (WT) and FMT-treated 5XFAD mice. (Right) Quantitative analysis of amyloids plaque in FMT-treated 5XFAD mice. Data are presented as mean ± SEM in percentage of young ("Young") group. Two slides per mouse were analyzed ($n$ = 9 for transgenic mice treated with cecal material from young and $n$ = 10 for mice treated with old donor material). Statistical analysis was performed by using Student's t-test (*, $p < 0.05$). Data are published in Valeri et al. (2021). Abbreviations: Young (treatment with cecal content from young wild type donors), Old (treatment with cecal content from old wild type donors), WT (wild type), ThT (Thioflavin-T).

The enhanced production of Aβ plaque deposition, rather than its processing, observed in the prefrontal cortex and dentate gyrus brain regions may allow speculation for a possible acceleration of AD pathogenesis due to transplantation of healthy but aged microbiota. Therefore, quantitative analysis of key molecules involved both in AD and in aging process was conducted by [redacted] (Department of Psychiatry and Psychotherapy, Johannes Gutenberg-University, Mainz; description of methods and result in Valeri et al. (2021)) in prefrontal cortex samples (Fig. 27 A).
Quantification of heterologous hAPP protein revealed no differences between 5XFAD young and old treated groups ($p$ = 0.7167) confirming that transplantation of aged microbiota increased the amount of plaque production but not of their precursor protein (Fig. 27 B). Therefore, it may be excluded that such elevation

in plaque deposition did not depend on *Thy1*, the promoter used to drive the *APP* overexpression and the generation of Aβ higher levels in 5XFAD mice (Oakley et al., 2006).
Among the investigated hallmarks, the enzyme NO synthase type I (NOS1) is involved in the production of nitric oxide (NO) that in turn, can react with superoxide radicals (produced by Aβ deposits in AD brains) inducing cellular injury (Galimberti et al., 2008). NO exerts a crucial function in maintaining the physiological homeostasis (Knott and Bossy-Wetzel, 2010; Tanaka and Seals, 2008) and its decrease in production, bioavailability and metabolic signalling is associated to decline in several physiological functions (Siervo et al., 2018; Sverdlov et al., 2014). Moreover, aging-related intestinal barrier dysfunction, represented by increased blood endotoxins levels and, generated by intraperitoneal administration of arginase inhibitor N(ω)-hydroxy-nor-l-arginine (norNOHA), resulted in lower expression of *inducible nitric oxide synthase* (*inos*) mRNA and $NO_2^-$ in small intestinal tissue of aged male (17 months) as compared to young (3 months) C57BL/6J mice (Brandt et al., 2022).
The glial fibrillary acidic protein (GFAP) is a marker for neuronal damage (known as astrogliosis) that is associated with higher Aβ plaque density in brain regions associated to AD (i.e., lower white matter integrity in the medial temporal lobe) and to impairment in episodic memory in AD patients (Bettcher et al., 2021; Chatterjee et al., 2021). Several studies support the idea, that the increase in GFAP expression is associated to higher astrocytes reactivity during aging, as provided by a conspicuous increase in the inflammatory genes (i.e., belonging to components C3 and C4B) observed in aged (2-year-old) mice in comparison to younger mice (see review Palmer and Ousman, 2018). Lack of knowledge concerns the role of NOS1 and GFAP proteins in the prefrontal cortex of 5XFAD mice receiving cecal content from young or old wild type donors. Therefore, analysis of these key proteins associated to AD and aging inflammation process was conducted, and no differences between the mentioned groups were detected (Fig. 27 C and D).
Brain-derived neurotrophic factor (BDNF) plays a fundamental role during brain development supporting the survival and differentiation of neuronal population (Bathina and Das, 2015). In addition, it is involved in the regulation of the synaptogenesis, synaptic transmission and plasticity (Molinari et al., 2020). Moreover, it exerts a crucial role in the maintenance of adult cortical neurons whose dysfunction contributes to the initial loss of short-term memory in AD (Giuffrida et al., 2018). Controversial findings have been reviewed concerning the role of BDNF during aging (Miranda et al., 2019). On one hand, circulating BDNF expression resulted reduced in blood samples of both aged primates and humans (Hayashi et al., 2001; Shimada et al., 2014) and in brains of rodents (Silhol et al., 2005). On the other hand, both the absence of changes (Lapchak et al., 1993; Narisawa-Saito and Nawa, 1996) and increase (Newton et al., 2005) in BDNF levels were observed in rodents' hippocampus during aging. However, human studies on BDNF are limited to serum analysis due to ethical reasons and a total lack of knowledge, concerns the *in vivo* BDNF analyses in the brain. The effect of gut microbiota on the brain BDNF levels have been reviewed (Maqsood and Stone, 2016). However, whether BDNF levels may also be affected by FMT from healthy young or old wild type in 5XFAD mice is an open question. Therefore, BDNF levels were investigated in the prefrontal cortex of 5XFAD mice subjected to cecal transplantation from young or old wild type donors. Analysis of BDNF levels, even if not statistically significant, showed a slight increase in the mice treated with cecal material from old wild type donors (Fig. 27 E).

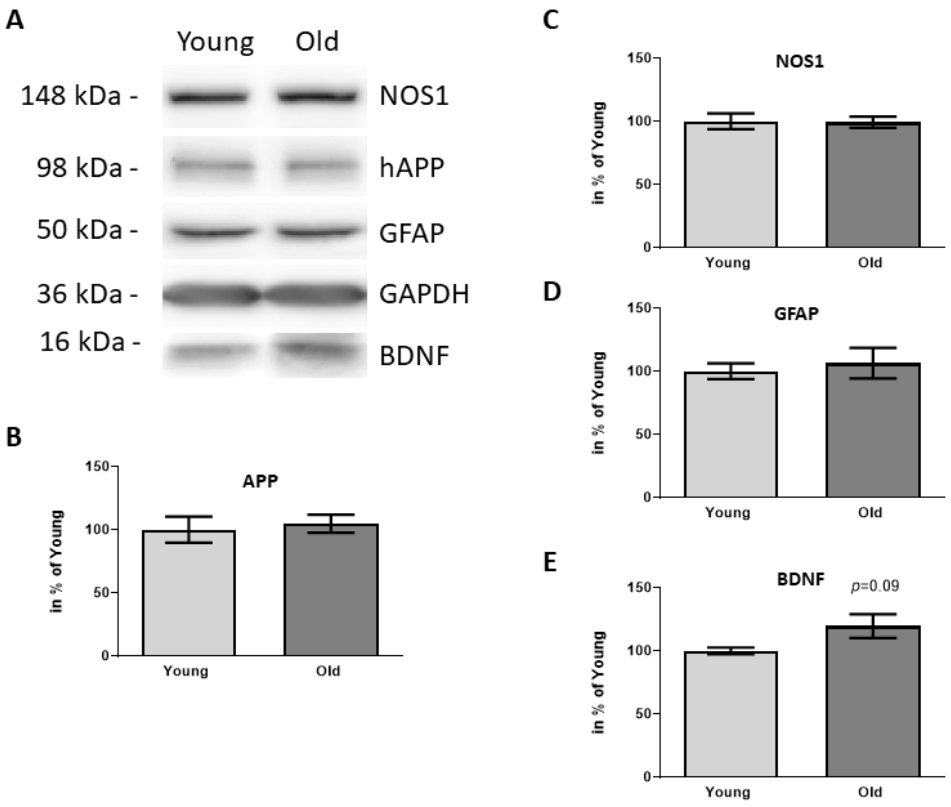



**Figure 27: Analysis of AD-related proteins in FMT-treated 5XFAD recipient mice.** The prefrontal cortex area was dissected and homogenized; 17.5 µg of protein were used for Western blot analysis. (A) Representative sample pair for each of the detected proteins is shown. (B-E) Quantitative analysis of AD-related proteins involved in the inflammation or neurotransmission processes (APP, NOS1, GFAP and BDNF). Data are presented as the mean ± SEM in percentage of the mean of animals treated with caecum content from young donors ("Young"). Sample sizes of $n$ = 6 - 7 per group were analyzed and normalized on GAPDH. Statistical analysis was performed by using Student's t-test (*, $p < 0.05$). Analysis performed by the [redacted] (Department of Psychiatry and Psychotherapy, Johannes Gutenberg-University, Mainz; data published in Valeri et al. (2021). Abbreviations: Young (treatment with cecal content from young wild type donors), Old (treatment with cecal content from old wild type donors), NOS1 (NO synthase type I), hAPP (human amyloid precursor protein), GFAP (Glial fibrillary acidic protein), GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase), BDNF (brain-derived neurotrophic factor).

In summary, increased Aβ-containing deposits were observed in prefrontal cortex and slightly in the hippocampus dentate gyrus slices of 5XFAD mice treated with cecal content from old healthy donors. It seems plausible that transplant of an old microbiota may exert its function evoking increasement in some of AD hallmarks independently from the used AD mice model (observation in both APPswe/PS1ΔE9 and 5XFAD mouse models). However, the reason for such significant increase in plaque deposition in the prefrontal cortex is unknown as many genetic and environmental factors may come into play (see chapter 1.3). Among the genetic factors influencing the Aβ production levels in AD patients, increased APP cleavage needs to be excluded as its levels were comparable between mice receiving cecal content from young and old donors (Fig. 27 A and B). In opposite direction from other reports showing increased microglia activation and NO production (induced in rodents by LPS injection at intraperitoneal level or directly in the brain (Brahmachari et al., 2006; Kang et al., 2019)), aged microbiota transplant seems to play a null effect on 5XFAD mice as shown by comparable levels of NOS1 and GFAP proteins between the two treatments (Fig. 27 C and D). However, lack of alteration in these proteins due to the received treatments in 5XFAD mice might hint for a more complex mechanism that involves the participation of inflammation proteins associated to the gut microbiota. Since Brandt et al. (2022) revelead a lower expression of *inos* mRNA and of $NO_2^-$ product in mice

aged 17 months, the observed lack of difference in NO production between the FMT-treated groups may be expected, as donor mice belonging to the old group were of 12 months of age.
Contrarily to other reports on aging showing BDNF deficiency in rodents aged brains or liquid biopsies (Palomer et al., 2016; Webster et al., 2002) and in AD pathological conditions (Ng et al., 2019; Silhol et al., 2008), slight higher BDNF levels were identified in the FMT-treated old group of mice. Whether BDNF levels in serum samples reflect the observed changes in the brain is a matter of debate. Indeed, several studies show a correlation (more than 75%) between the measures conducted in central nervous system with the ones in serum samples, pointing to the general idea of BDNF being able to cross the BBB (Klein et al., 2011; Rasmussen et al., 2009; Sartorius et al., 2009). This might hint to a more accessible alternative way of investigation for humans in which, analysis of BDNF in serum may be used as a proxy for the brain (Di Lazzaro et al., 2007; Pan et al., 1998; Pardridge et al., 1998). Moreover, higher BDNF levels were also detected in human serum samples at the very early stages of AD suggesting a compensatory repair mechanism in early neurodegeneration that could contribute to increase the degradation of Aβ (Laske et al., 2006). Therefore, a deeper analysis on serum BDNF levels is required in future studies concerning the role of FMT in 5XFAD mice that received cecal content from old and young healthy donors. In addition, studies in older mice (more than 10 weeks) and with prolonged chronic FMT treatment (more than 6 weeks) may differently affect the final levels of BDNF giving a more pronounced statistical significance between treated groups. Based on these studies, it is also possible to assume that cecal material from the old donor mice started a vicious development that may diverge overtime.
The existing differences between AD mouse models and patients may compromise the translatability of results obtained in mouse studies towards humans (see chapter 1.8.3 for translational issues). In particular, microbiota composition changes between mice and humans due to difference in their anatomy and other confounding factors (e.g., age, sex, diet, comorbidities, use of medication and inclusion of small sample sizes in human studies) (van Olst et al., 2021). At technical level, most of the research uses transplant of cecal content in mouse, while human studies use stool samples (van Olst et al., 2021). In addition, the used transgenic 5XFAD mouse model presents mutations associated to familial AD leading to of an excessive production of Aβ pathology, while the vast majority of AD cases in humans are sporadic (see chapters 1.3 and 1.5). Therefore, the selection of a suitable donor for both mouse models and humans remain a demanding task. In humans, donors should be chosen based on their good health condition (e.g., no predisposition to genetic disease, including diabetes and obesity) and absence of any detectable infectious agents (Ianiro et al., 2022; Junca et al., 2022). Moreover, donors need to be carefully selected since, molecular changes in biomarkers (i.e., Aβ plaques and NFTs) associated to AD start to begin more than two decades before the onset of clinical symptoms in human pathology (Fortea et al., 2020; Holtzman et al., 2011). Another possibility that may bridge the differences between mice and humans concerns the use of mice with humanized microbiota (Goodman et al., 2011). However, generation of such mice is extremely hard to produce as several factors come into play when translating to human gut microbiota (e.g., anatomical and physiological factors, diet and environmental stimuli) (Park and Im, 2020). Moreover, alteration of microbiota composition in AD, observed between humans and animal models, may also depend on different disease stages (Brandscheid et al., 2017; Harach et al., 2017; Haran et al., 2019; van Olst et al., 2021; Zhuang et al., 2018). In conclusion, despite translational issues, acute administration of aged microbiota into young recipient 5XFAD mice was able to reproduce certain aspects common to healthy human elderly and healthy aged mice already after 6 weeks from treatment. In addition, selective AD hallmarks resulted aggravated in mice after having received cecal content from old healthy mice. It may be possible to assume that a prolonged chronic treatment may shed light on unknown molecular and behavioral mechanisms that were not covered by the acute administration.

## 4.8 Activation of enteric neurons in Arc-GFP mice subjected to chronic social defeat stress paradigm

Stress is an environmental factor that is associated to acceleration of AD in rodents (review Justice, 2018). Among the AD hallmarks involved, increased *APP* expression and Aβ production was observed in rodents upon both acute and chronic stress exposure, from mild to intense stress magnitudes (Briones et al., 2012; Justice, 2018; Ray et al., 2011; Rosa et al., 2005; Sayer et al., 2008; Solas et al., 2010). In addition, both levels of hyperphosphorylated tau and NFTs resulted elevated by stress in AD mouse models (Feng et al., 2005; Fujio et al., 2007; Korneyev, 1998; Kvetnansky et al., 2016; Okawa, 2000; Rissman et al., 2007). At behavioral level, loss in cognitive performance was accelerated in AD mouse model subjected to stress exposition (Dong et al., 2004; Jeong et al., 2006). Although several studies conducted in animals correlate the stress exposure to a worsening of the pathology, this was not proven in humans as lifestyle changes between individuals and several other factors may come into play (review Justice, 2018). In the last decade, the role of stress has been emerged in relation to the microbiome and to the gut dysbiosis (review by Dogra et al., 2020). The gut microbiota is involved in the processes leading to the intestinal permeability and to the final establishment of a "leaky gut" (see chapter 1.7.1; Fig. 3B). Such processes were mediated by physiological and behavioral consequences of stress exposure, that include alteration in the HPA axis (Gareau et al., 2007; Sudo et al., 2004), compromised cognition (Gareau et al., 2011), enhanced inflammation (Bailey et al., 2011; Maslanik et al., 2012), compromission of the intestinal barrier function (Bailey and Coe, 1999; Gareau et al., 2007; Mackos et al., 2016; Söderholm and Perdue, 2001; Zheng et al., 2017, 2013) and alteration in social behavior (Bailey and Coe, 1999). Therefore, intestinal microbiota may act as a mediator of chronic stress responses (Karl et al., 2018; Mackos et al., 2016; Sudo et al., 2004). Different kinds of stressor events negatively impact the gut microbiota composition in several animal studies (e.g., maternal separation and chronic restraint stress induce a reduction of *Lactobacilli*) (Bailey et al., 2011; Bailey and Coe, 1999; De Palma et al., 2014; Karl et al., 2018; Tannock and Savage, 1974; Zheng et al., 2013). The capacity to adapt to the overcoming stress and adversity maintaining the normal psychological and physical functions is defined as "resilience", while the opposite as "susceptibility" (Russo et al., 2012; Rutter, 1993; Southwick et al., 2005). Several animal models were used to address such heterogeneity of response to stress basing on the chronic social defeat stress (CSDS) paradigm (Krishnan et al., 2007; Yoshida et al., 2021). In detail, animal protocols for CSDS paradigms have already been reviewed (see Hollis and Kabbaj, 2014). In most of the CSDS paradigms, mice can be classified either resilient or susceptible based on their exposition to administration of 10 daily bouts with an aggressor male mouse with the CD-1 strain (Calvo et al., 2011; Larrieu et al., 2017; Yohn et al., 2019). Susceptible mice exhibit alterations at behavioral, neural, and hormonal levels according to the received chronic stress and to the social avoidance towards the aggressor, whereas resilient mice do not show these alterations and remain social interested towards the aggressor (Dos Santos Guilherme et al., 2021; Krishnan et al., 2007; Krishnan and Nestler, 2008; Yohn et al., 2019). Changes in the fecal microbiome have been addressed in C57BL6/J mice subjected to CSDS paradigm (Bharwani et al., 2016). In particular, male mice exposed to an aggressor mouse with the CD-1 strain for 10 days displayed reduced microbial richness and diversity (Bharwani et al., 2016).

As previously reported in chapter 1.7, the GIT exerts several complex digestive functions associated to gut microbiota. In addition, within the GIT and embedded in the gut wall, resides the ENS, a complex intrinsic neural network responsible for the coordination of multiple functions, including the response to stress events (Gershon and Erde, 1981; Million and Larauche, 2016; Wood, 2008). Moreover, CNS and ENS are bidirectionally connected in the well-known GBA. On one hand, signals induced by the perception of stressful events are directed by the brain to the ENS through the efferent fibers of the vagal nerve (Breit et al., 2018; Tubbs et al., 2015). On the other hand, afferent fibers of the vagal nerve are implicated in the stimulation of HPA axis which, in turn, directs the adaptive stressors responses of the organism (Breit et al., 2018; Howland, 2014; Tubbs et al., 2015).

To shed light on the link between stress experiences and the activation of enteric neurons, a transgenic Arc-sfGFP mouse model was used in this study (model described in Guenthner et al., 2013). This mouse model was based on the *Arc* promoter activity to enable GFP expression in enteric neurons upon Tamoxifen administration (Guenthner et al., 2013).

Male and female Arc-sfGFP mice aged 4 weeks were exposed to a CSDS paradigm to induce a stratification into resilient and susceptible subgroups based on behavioral profiles. The *Arc* promoter activity has been

assessed after 5 (for males) and 9 (for females) hours from the Tamoxifen intraperitoneal injection analyzing the GFP-positive neurons cell counts in the *myenteric plexus* (a branch of ENS within the outer LMMP and derived from ileum) by fluorescence microscopy (Fig. 28 A). Interestingly, a significantly higher number of GFP-positive neurons cell counts was detected both in male and female mice that were exposed to chronic social defeat before assessment of social interaction (labeled as "stressed") compared to their respective control mice that were not exposed to chronic social defeat but only to social interaction (methods described in Chongtham et al., 2021; Dos Santos Guilherme et al., 2022). No differences were observed between resilient and susceptible stratified subgroups regardless of sex (Fig 28 B and C). However, baseline numbers of activated enteric neurons were different between control male and female Arc-sfGFP mice with higher levels in the latter (females: 78 ± 9 versus males: 45 ± 3; $p = 0.017$) (Fig 28 D and E). This may suggest the presence of sex dimorphism in enteric neurons residing in LMMP.

In summary, a tremendous activation of enteric neurons has been observed in both male and female mice subjected to stressful events. A possible explanation for the observed increased neuronal activation may reside in the presence of a mutual exposition between the ENS and the gut microbiota due to the GBA (chapter 1.7). Taking into account the proximity of the microbiota to the ENS, microbes harboring the gut can either directly or indirectly influence its development and function (Hyland and Cryan, 2016). In particular, bacteria residing in the gut are involved in the host production of several signaling molecules, including neurotransmitters (e.g., serotonin) and hormones that modulate the ENS of adult mice (De Vadder et al., 2018). The role of *Arc* has not come into focus in relation to enteric neurons or stressful events. However, it has been shown that Arc protein was highly expressed in hippocampal CA1 neurons and, this was correlated with behavioral response induced by chronic stress in mice (Leem and Chang, 2017). Additionally, due to its expression in neurons, *Arc* has been used as technical tool to address hippocampal expression patterns of resilient and susceptible mice (Dos Santos Guilherme et al., 2022). Moreover, *Arc* expression was also associated to increased EphB2 signaling in irritable bowel disease model rats and to synaptic plasticity (L. Zhang et al., 2022). Therefore, it is plausible that different stressors may modulate the *Arc* expression. This allows speculation on the involvement of *Arc* as a possible mediator in the GBA between the gut microbiota changes and the activation of enteric neurons.

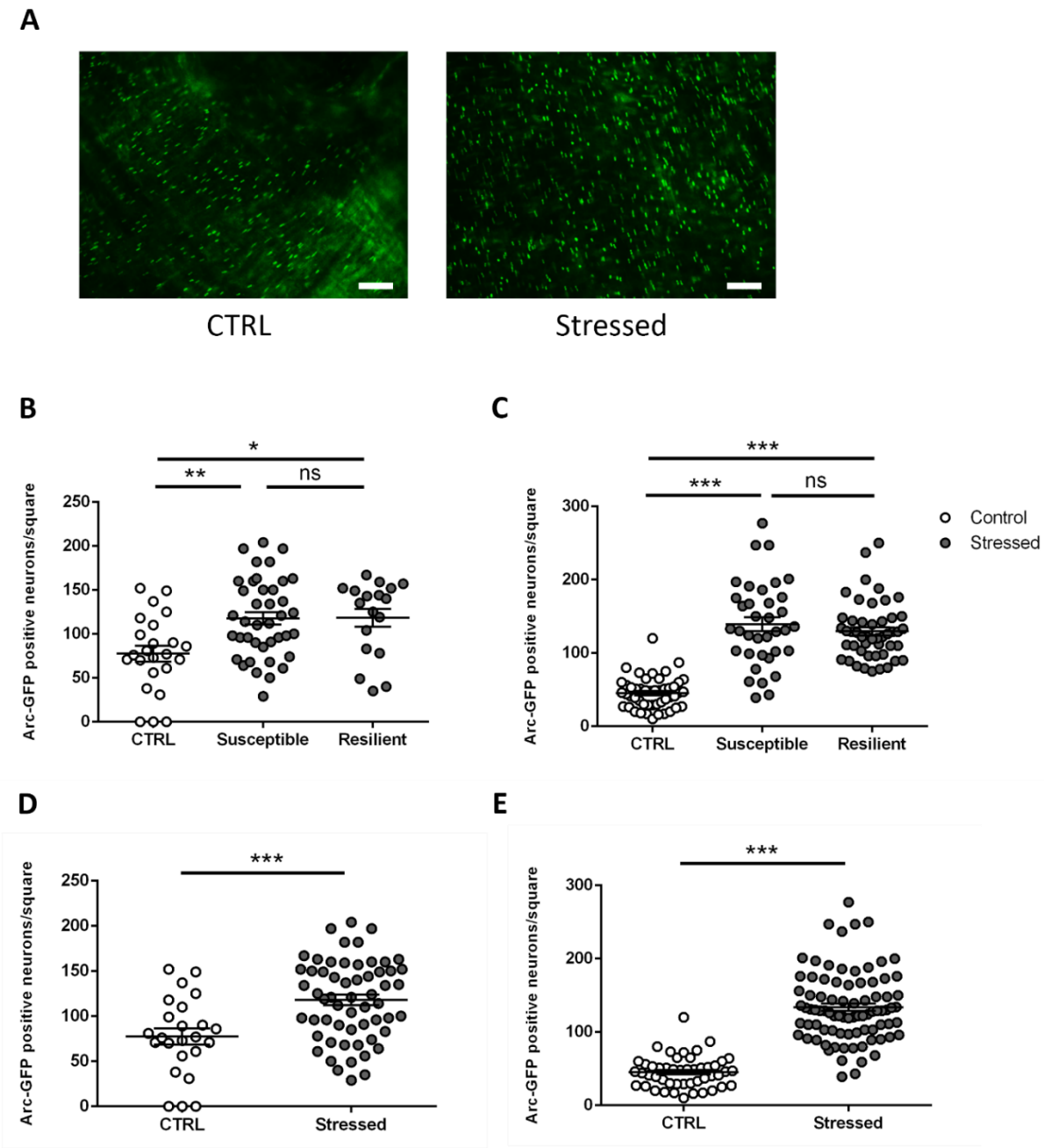



**Figure 28: Analysis of activated enteric neurons residing in the ileum myenteric plexus in male and female Arc-sfGFP mice subjected to CSDS paradigms.** (A) Representative picture of enteric neurons expressing Arc promoter activity via sGFP expression in the LMMPs of mice subjected to defeated (stressed) and undefeated control (CTRL) mice. Tamoxifen was administrated 5 h (for males) and 9 h (for females) before the social interaction test label activated neurons (methods described in Guenthner et al., 2013). Scale bar: 100 µm. sGFP positive neurons were counted from defined areas for each mouse (see Fig. 6) in female (B and D) and male (C and E) mice. (B) Female mice: sGFP positive neurons were counted in three squares from two independent images per mouse ($n$ = 4 for resilient, 7 for susceptible and 5 for control). (C) Male mice: sGFP positive neurons were counted in three squares from three independent images per mouse ($n$ = 4 for resilient, 3 for susceptible and 4 for control). (D and E) Data from susceptible and resilient mice belonging to B and C were pooled in the stressed group. Data layout from (B) and (D) were slightly modified from published article of Dos Santos Guilherme et al. (2022), while (C) and (D) consist of unpublished data. Statistical analysis was performed by one-way ANOVA (Tukey's multiple comparison test; (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Abbreviations: Arc-sfGFP (Mouse model: ARC-creERT2/+.R26CAG-LSL-Sun1-sfGFP-Myc/+), CTRL (undefeated control), ns (not statistically significant).

# 5. Conclusion and future perspective

In the here presented dissertation, the effect of cecal microbiota transplant from wildtype mice donors into recipient transgenic 5XFAD mice was investigated in relation to aging. A modified antibiotics treatment regime, based on Minter et al. (2016), was able to reduce the baseline CFUs level of bacteria in the gut, allowing the grafting of external donors' microbiota in recipient 5XFAD mice. Then, transplant of an aged cecal microbiota provided by healthy wild type donors into recipient mice was evaluated by monitoring the levels of the chosen representative target families of Enterobacteriaceae and Lactobacillaceae and by specific group of bacteria through qPCR analysis up to 6 weeks after inoculation. The choice of the described target bacteria families was based on their effect related to the aging process and on their opposite function on the host homeostasis (see chapter 1.9). Interestingly, higher Lactobacillaceae levels were detected in 5XFAD mice treated with cecal material from old donors after transfer, showing homogeneity with the results obtained in old donors. However, qPCR analysis revealed the lack of differences for a wider selection of bacteria among the treated groups except for general Firmicutes phylum and species belonging to *Bifidobacterium* genus. Indeed, decrease of different *Bifidobacteria* species were detected in 5XFAD mice treated with cecal material from old donors compared to the ones treated with cecal material from young donors confirming previous reports in human adults and elderly (see Table 2). The use of a germ-free line of mice would be an alternative step of validation for the efficiency of FMT treatment instead of the already used antibiotics treatment. A future investigation would be to expand such analysis to fecal microbes that were not covered by qPCR. Indeed, only 0.1–10% of microbial species can be differentiated using the here used techniques. Therefore, next generation sequencing (NSG) may be used to remove the limitations and boundaries associated with classic culture-based approaches (Malla et al., 2019; Wu et al., 2017). Moreover, future research on fecal transplant from human adults and elders to 5XFAD mice may clarify whether the observed changes in bacterial specimens are unique for mice or if can also be translated to humans. The stability of donors' microbiota in recipient mice was assessed up to 6 weeks after transplant confirming consistency with a previous study (Ellekilde et al., 2014). However, longer distance studies (more than 6 weeks) may confirm whether the microbiota stability will be maintained and/or if other microbial communities may change over time.

The proximity effect of the FMT was investigated on the host duodenal morphology. Interestingly, 5XFAD mice treated with cecal content from old donors showed increased villus length as well as elevated submucosa and muscularis thickness reflecting previous observations on aged mice (El-Salhy et al., 1999; Nalapareddy et al., 2017). These evidences suggested, that the implant of external microbiota provided by old donors into the host, exert a critical role in duodenum morphology. Presumably, such alteration in the duodenal structures may be caused by a decline in tissue regenerative capacity occurring during aging. Since intestine is a stem cell-based organ (Nalapareddy et al., 2017), analysis of intestinal stem cells (ISCs) and underlying pathways (e.g., downregulation in canonical Wnt signaling during aging and AD in rodents (Palomer et al., 2019)) should be carried out in 5XFAD mice treated with cecal material from old donors. In addition, alteration of mRNA expression levels of genes, involved in Glucagon-like peptide-2 signaling pathway (e.g., *Glp-2r* and *Igf-1*), were associated to altered the villus morphology and in nutrient's digestion/absorption in aged SAMP mice (Suzuki et al., 2022). Therefore, whether the observed changes in duodenal morphology structures, due to transplant of cecal content from old donors in 5XFAD mice, may be associated to alteration of such genes is unknown and future studies should be carried out as a support analysis. Aged mice are also characterized by compromised nutrient absorption, whose function is mainly exerted by enterocytes residing in the microvilli brush border (Barzilai et al., 2012; Yoshimoto et al., 2021). Increased rate of enterocytes apoptosis, shown by higher levels of cleaved caspase-3 positive cells, together with reduced enterocyte maturity and absorptive efficacy, have been observed in intestinal crypts and villi of aged mice (Ciccocioppo et al., 2002; Moorefield et al., 2017). Therefore, whether aged microbiota is able to induce such increase rate of apoptosis, normally associated to the aging process, also in transplanted 5XFAD mice is a further analysis to be covered in future experiments. Among the functions exerted by gut microbiota, the intestinal barrier functions and motility resulted altered during aging (Orr and Chen, 2002; Wilms et al., 2020). In addition, some studies suggested that such impaired functions may be attributed to increased muscle layer thickness in older animals (Wilms et al., 2020). In accordance with this hypothesis, in

the current investigation, transgenic mice treated with FMT from old donors showed a similar result (i.e., increased submucosa and muscularis thickness), suggesting that transplantation of an aged microbiota may induce an aging-associated phenotype. However, future investigation on the mechanisms at the base of contractile properties (e.g., myosin phosphorylation and calcium signalling pathways) of intestinal muscle in relation to FMT should fill this gap. Furthermore, analysis of gut transition time, normally affected in aged rodents (Diss et al., 2013; Smits and Lefebvre, 1996), may be a support analysis to confirm whether changes in the muscular thickness may be associated to a transplant of aged microbiota. Furthermore, analysis of nutrients intake, normally altered in aged mice due to compromised gut structure, should be evaluated as an additional confirm of the effect of the microbiota transplant.

A common phenotype associated to aging and elderly is represented by chronic inflammation process and altered nutrient intake that may induced medical complications including diabetes and inflammatory bowel disease (Hunter et al., 2010; Seo and Holtzman, 2020). Therefore, whether transplantation of aged donor microbiota may induce similar changes, several physiological parameters, known to be affected by aging, were investigated. Among those, a tendency to increase in abdominal fat amount, increased serum LBP concentration, and decrease in body temperature were also observed in relation to the aging process. However, among the physiologic factors that were not affected by FMT, same levels of blood sugar concentration were observed between the two groups of treatment. As already discussed before (see chapter 4.6), the described lack of differences in blood sugar concentration may be attributed to the method adopted such as the antibiotic treatment. Nevertheless, conflicting results have been reported in regard to the glucose absorption levels between young and old individuals in both human and animal studies (see review Saffrey, 2014). Prolonged chronic FMT procedure (more than 6 weeks) should be carried out in order to assess whether sugar concentration may change between the two treatments and, whether abdominal fat may generate significantly increase in the old group of mice. Furthermore, density analysis of the $Na^+$-dependent glucose transport (e.g., SGLT1, GLUT2 and $Na^+K^+$-ATPase), known to be involved in the glucose uptake (see review Drozdowski and Thomson, 2006), should clarify whether the transplant of cecal content may be involved in changes associated to blood sugar concentration. Support analyses of some variables associated to the regulation of lipid metabolism, such as cholesterol, insulin and glucose (e.g., mRNA expression of genes involved in cholesterol synthesis, including *GLUT2*, *GK*, *SREBP2* and *HMGCS)* are also required in FMT-treated mice. Moreover, lipid metabolism resulted altered in aged mice receiving young donor microbiota and, such alteration was associated to microglial function (Parker et al., 2022). Among the fatty acid exerting anti-inflammatory function on microglia, increased levels of palmitate were also observed in aged C57BL76 mice treated with microbiota from young donors (Kim et al., 2019; Melo et al., 2020). Therefore, whether changes in specific metabolites occur also in 5XFAD mice subjected to cecal content donation from healthy old mice, should be supported by further investigation.

Loss of thermoregulation was assessed with infrared thermometer and associated to better nest building in mice treated with cecal content from old donors. Moreover, activation of GABAergic neurons in the ventrolateral preoptic nucleus (vLPO) of hypothalamus was associated to reduced body temperature (Zhao et al., 2017). Therefore, use of markers for neuronal activity (e.g., *c-Fos* expression) in vLPO area between mice treated with cecal content from young and old donors would further confirm whether FMT induce activation of neurons associated to decline of body temperature. The drop in body temperature was associated to a tendency to build better quality nest in mice treated with cecal material from old donors. Despite these small changes obtained in the nest building ability, other behavioral tasks to address the effect on body temperature should be conducted in older (more than a final age of 10 weeks) FMT-treated mice. Among those, huddling test, a behavioral mechanism for conserving the body temperature (Fontaine et al., 2018), would be a further supporting tool to confirm the obtain differences in the nesting test. Moreover, no differences between treatments were observed in the investigated behavioral tasks (i.e., Neophobia test, T-maze and RAWM) associated to AD. However, differences in behavioral tasks were not expected in general in 5XFAD mice, since deficits, due to the 5XFAD mouse strain, start to appear around 4–6 months of age (see chapter 1.5). Therefore, in future experiments, re-evaluation of behavioral tasks should be conducted in older mice (more than a final age of 10 weeks) with a more prolonged post-inoculation phase or with repeated FMT treatments. In addition, whether aged microbiota transplant may or may not show a deleterious effect in spatial learning and memory also in 5XFAD mice will be object of further investigations. To date, conflicting results in behavioral tests (i.e., Morris Water Maze) have been reported in young C57BL/6 mice that received

microbiota from aged mice (Boehme et al., 2021; D'Amato et al., 2020; Parker et al., 2022), suggesting the presence of a subtle mechanism at the base of the behavioral phenotype that need to be unveiled.
FMT treatment revealed a worsening of AD pathology in mice treated with cecal content from old donors evaluated by increased plaques deposition in the prefrontal cortex and hippocampus brain areas. Reasonably, such increased plaque deposition was not dependent on *Thy*-driven promoter activity related to *APP* gene as revealed by its lack of difference in protein levels between the two treatments. In addition, inflammation markers, such as NOS1 and GFAP proteins, known to be involved in the neuroinflammation process and in AD pathology, did not show any sign of difference in the prefrontal cortex samples between the received treatment. As already discussed in chapter 4.7, additional analysis on the expression levels of *inos* mRNA and $NO_2^-$ product levels, known to be reduced in aged mice, were not investigated, and future studies needs to cover this gap in relation to FMT and inflammaging in recipient transgenic AD mouse models. However, higher LBP levels were detected in serum samples of old group of mice. Whether inflammation is just about to rise or if it is in an ongoing process, this should be addressed by future experiments evaluating the LBP levels in AD-associated brain regions. Additional prolonged incubation with FMT would clarify if the increased LBP levels are limited only to the organism periphery or not. Aside LBP, other aged-associated biomarkers for intestinal permeability ("leaky gut") and altered gut morphology, such as tight junctions (e.g., occludins, claudins, junctional adhesion molecules (JAM), tricelluins and zonula occludens (ZO) proteins) should be investigated. Among the mediators involved in the inflammation process for their properties of crossing the BBB, cytokine signalling and immunomodulatory factors (e.g., IL6 and Tumor necrosis factor α (TNF- α)) were known to be affected by gut microbiota products (Ha et al., 2020; Han et al., 2019), and therefore should be investigated in relation to the aging process in FMT-treated mice. Additionally, whether FMT is involved in the microglia activation, at the base of the inflammaging state, in brain regions affected to AD is still unclear. Recently, Parker et al. (2022) showed increased level of Iba-1 positive cells, a marker for microglial activation, in the corpus callosum and the cortex in young mice treated with microbiota transplant from old donors, while opposite result was obtained by transplanting young donor microbiota in aged recipients (Parker et al., 2022). Therefore, whether transplant of cecal content from old donors may or may not aggravate the activation of microglia in the investigated brain areas of 5XFAD was not addressed so far. Future investigation on microglia would define if the obtained lack of changes in the inflammation markers (i.e., NOS1 and GFAP proteins) between the two group of treatment may be attributed to a too young age of the transplanted cecal microbiota mice (5XFAD mice with final age of 10 weeks) or if other known mechanisms may be involved.
Although decreased levels of BDNF were reported in serum and brain samples of aged mice and patients or affected by AD pathological condition (Ng et al., 2019; Silhol et al., 2008; Webster et al., 2002), a slight increase in BDNF levels was detected in the prefrontal cortex of mice treated with cecal material from old donors. Such deviation from previously reported studies could be explained by the early age (final age 10 weeks) of investigated animals. Nevertheless, in accordance with Laske et al. (2006), increased serum BDNF levels were also detected in patients with early stages of probable AD in comparison to patient in a more severe stage. Therefore, it is possible to assume that the transplant of aged microbiota may contribute to ameliorate the BDNF-based repair mechanisms in AD early neurodegeneration. Another possibility contemplates the presence of an age-dependent vicious mechanism - just at its rise - started by old cecal material transplant. Therefore, prolonged FMT incubation and the use of older recipient 5XFAD mice may pave the way on the inflammation-based mechanisms in relation to the aging process. Moreover, TrkB (tropomyosin-related kinase B) receptors, known to mediate the effect of BDNF in the synaptic transmission and LTP in the hippocampus and in other brain regions (Leal et al., 2014), should be evaluated in future experiments.
Whether all these findings may be applied to AD patients is still an open question due to the high complexity of their microbiota and genetics. Indeed, there is a high possibility that AD patients subjected to FMT provided by healthy human donors may develop infections that may aggravate their pathology-associated symptoms. To date, standard microbiological screening tests may be used as a possible alternative to avoid the risk of infections connected to the transfer of microorganism into the host, especially for those linked to antimicrobial resistance and to diseases (e.g., *Fusobacterium nucleatum* and *Sutterella* species are involved in IBD and cancer)(Giles et al., 2019). Another future challenge will be to develop appropriate microbial screening strategies that take into account also different factors (i.e., genetics, immunity and environment)

implicated in both AD and gut microbiota. This will open the way to future medical treatments that are based on the selection of mixtures of microorganisms, or of their related products, able to define a more reliable therapy based on optimal treatment regime and risk profiles.

Among the environmental factors implicated in sporadic AD, chronic stress exposure is associated to acceleration of the pathology in humans and rodents. Transgenic male and female Arc-sfGFP mice, subjected to chronic social stress paradigm (stratified in resilient and susceptible to stress), revealed increased activation of enteric neurons in the LMMP of *ileum* upon Tamoxifen administration compared to control animals not subjected to chronic social stress paradigm. An intriguing question concerns the possibility that gut microbiota may be implicated in such massive neuronal activation in stressed mice. However, gut microbiota manipulation (i.e., mice treated either with antibiotics or probiotics) did not alter the mice CSDS-associated behavioral outcome (Dos Santos Guilherme et al., 2022) suggesting the presence of alternative factors contributing to such elevated neuronal activation in the ENS.

An interesting point to address would consider whether the effect of the gut microbes' products may have an impact on the enteric neurons' activation and on the related pathways. Therefore, evaluation of signaling molecules produced by gut bacteria (e.g., neurotransmitters and metabolites) on ENS neurons should be addressed. Indeed, it is well known that local neurotransmitters, such as GABA, serotonin, melatonin, histamine and acetylcholine, together with active catecholamines and SCFAs metabolites produced by gut microbiota, exert their function influencing the ENS activity and with this, memory and learning in mice (review Carabotti et al., 2015). However, the mechanisms behind the interconnection between the microbiota and the host neuronal activity and behaviors (e.g., social activity, stress and anxiety responses) is poorly understood (Chu et al., 2019). Therefore, future investigations need to clarify whether such factors may modulate the enteric neuronal activation upon CSDS paradigm and if the result of such changes may have consequences on the CNS pathologies, including AD. Moreover, *c-Fos* expression, a marker for neuronal activity, was increased in different brain regions (i.e., paraventricular nucleus of the hypothalamus, bed nucleus of stria terminalis and hippocampal dentate gyrus) associated to stress response in germ-free and antibiotic-treated mice after being exposed to social encounter (W. L. Wu et al., 2021). Hence, aside the use of *Arc* as technical tool of investigation, assessment of *c-Fos* expression in enteric neurons and brain regions associated to stress should be addressed in mice subjected to CSDS paradigm.

Finally, whether gut microbiota and enteric neurons react independently or in a cooperative manner to stress exposure is unknown and the goal of future research is to close this gap.

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## 7. Curriculum Vitae

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