Diploma thesis

DYSBIOSIS, GUT BARRIER DYSFUNCTION, AND INFLAMMATION IN DEMENTIA

submitted by

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Graz, 09 November 2022

Affidavit

I hereby declare that the following diploma thesis has been written only by the undersigned and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of this thesis other than those indicated in the thesis itself.

Graz, 09 November 2022 *Lara Engertsberger eh.*

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Abbreviations

A

Aβ	amyloid β
Ac	acetyl coenzyme A
AD	Alzheimer’s disease
AICD	amyloid precursor protein intracellular domain
ANCOM	Analysis of Composition of Microbiomes
APOE	Apolipoprotein E
APP	amyloid precursor protein
ASV	amplicon sequence variants
ATP	adenosine triphosphate

B

BACE1	β-site APP-cleaving enzyme 1
BBB	blood brain barrier
BFCNs	basal forebrain cholinergic neurons
BLAST	Basic Local Alignment Search Tool
BMI	body mass index

C

C83	α-C-terminal fragment
C99	β-C-terminal fragment
CD	cluster of differentiation
CDR	Clinical Dementia Rating
CRP	C-reactive protein
CSF	cerebrospinal fluid
CTF	C-terminal fragment

D

DAO	diamine oxidase
DM	diabetes mellitus
DNA	deoxyribonucleic acid

E

ELISA	enzyme-linked immunosorbent assay
ETEC	enterotoxigenic *Escherichia coli*
EU	endotoxin units

F

Faith PD	Faith’s phylogenetic diversity
FDR	false discovery rate
FFAR3	free fatty acid receptor 3

G

GABA	γ-amino butyric acid
GF	germ-free
GPR	G protein-coupled receptor

H

HIV	Human immunodeficiency virus
HSV	Herpes simplex virus

I

IBD	inflammatory bowel disease
ICD-10	International Classification of Diseases 10
IgG	immunoglobulin G
IL	interleukin

K

KEGG	Kyoto Encyclopedia of Genes and Genomes

L

LBP	lipopolysaccharide binding protein
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis Effect Size
LPS	lipopolysaccharide

	M
MMSE	Mini Mental State Examination
MNA-SF	Mini-Nutritional Assessment Short-Form
	N
NF-κB	nuclear factor kappa-light-chain-enhancer of the signalling pathways of activated B cells
NFTs	neurofibrillary tangles
NGF	nerve growth factor
NLRP3	NLR family pyrin domain containing 3
NSAIDs	non-steroidal anti-inflammatory drugs
	O
OTU	operational taxonomic unit
	P
PC	principal components
PCoA	Principal Component Analysis
PCR	polymerase chain reaction
PERMANOVA	Permutational Multivariate Analysis of Variance
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PPAR	peroxisome proliferation activated receptor
PPIs	proton pump inhibitors
	R
RDA	Redundancy Analysis
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
	S
sAPP	soluble amyloid precursor protein
sCD14	soluble cluster of differentiation 14
SCFA	short chain fatty acid
sp.	species
	T
T1DM	Type 1 DM
T2DM	Type 2 DM
T_H	T helper cell
TLR4	Toll-like receptor 4
TNF	tumour necrosis factor
T_{reg}	regulatory T cell
TSS	total sum scaling
	V
VIF	variance inflation factors

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Already released publications

Engertsberger L, Horvath A, Komarova I, Feldbacher N, Leber B, Pichler G, et al. Dysbiosis in dementia is associated with gut barrier dysfunction and linked to malnutrition and prescription drug use. Z Gastroenterol. 2020;58(05): P29. https://doi.org/10.1055/s-0040-1712280.

Stadlbauer V, Engertsberger L, Komarova I, Feldbacher N, Leber B, Pichler G, et al. Dysbiosis, gut barrier dysfunction and inflammation in dementia: a pilot study. BMC Geriatrics. 2020;20(1): 248. https://doi.org/10.1186/s12877-020-01644-2.

Zusammenfassung

Hintergrund: Demenz ist eine weltweit zunehmende Bedrohung für das Gesundheitswesen, deren Pathogenese noch nicht vollständig geklärt ist. Entzündungsprozesse stehen im Verdacht, den kognitiven Abbau voranzutreiben. Was diese Inflammation verursacht, ist bis dato jedoch unbekannt. Unserer Hypothese nach resultieren Störungen der Zusammensetzung des Darmmikrobioms in einer Dysfunktion der Darmbarriere und der Wanderung von Bakterien über den Blutkreislauf bis ins Gehirn. Die dadurch ausgelöste Entzündungskaskade könnte neurodegenerative Prozesse begünstigen.

Methoden: Um diese Hypothese zu testen, wurde eine Kohorte von 23 Demenzpatient*innen und 18 alters- und geschlechtsangepassten Kontrollpersonen ohne kognitive Beeinträchtigungen untersucht. Zusammensetzung des Darmmikrobioms, Darmbarrierestörung, bakterielle Translokation und Inflammationsstatus wurden anhand von Stuhl- und Serumproben bewertet. Mittels Mini Nutritional Assessment Short Form (MNA-SF) wurden Mangelernährungszustände erfasst, zusätzlich wurde die aktuelle Medikamenteneinnahme dokumentiert. Stuhlmikrobiota wurden 16S-sequenziert und mittels QIIME 2- und Calypso 7.14-Tools analysiert.

Ergebnisse: Demenzpatient*innen wiesen eine dysbiotische Mikrobiomstruktur auf, die durch Unterschiede in Beta-Diversität und Veränderungen in der taxonomischen Zusammensetzung gekennzeichnet war. Butyrat-produzierende Bakterien, darunter *Eubacterium rectale* und Mitglieder der Gattung *Lachnospiraceae*, waren bei dementen Personen in geringerer Anzahl vorhanden. Demenzpatient*innen litten unter gesteigerter Darmpermeabilität, indiziert durch hohe Serum-Diaminoxidase-Werte. Erhöhte Serum-Spiegel von löslichem Differenzierungscluster 14 (sCD14) bestätigten die Präsenz systemischer Entzündungsprozesse. In einem multivariaten Modell konnten die Mikrobiomveränderungen in Demenzpatient*innen am besten durch den Einfluss von Body mass index (BMI) und der Einnahme von Statinen erklärt werden. Bemerkenswerterweise nahmen demente Patient*innen dreimal mehr verschreibungspflichtige Medikamente ein als Personen der Kontrollgruppe. Drei Viertel aller Demenzpatient*innen waren unterernährt.

Schlussfolgerung: Demenz korreliert mit einer veränderten Darmmikrobiom-Zusammensetzung und erhöhten Biomarkern für Darmpermeabilität und Entzündung. Im Darmmikrobiom dementer Personen finden sich weniger Butyrat-produzierende Bakterien. Dies lässt vermuten, dass Butyratmangel das fehlende Puzzleteil zwischen intestinaler Dysbiose, Darmbarrierestörung und Neurodegeneration bildet. Darüber hinaus beeinflussten

Mangelernährung und Medikamenteneinnahme die Zusammensetzung des Mikrobioms von Demenzpatient*innen. Daher stellen die Substitution von Butyrat-produzierenden Bakterien und die Bekämpfung von Mangelernährung vielversprechende zukünftige Therapieziele bei Demenz dar.

Abstract

Background: Dementia is an increasing public health threat worldwide. The pathogenesis of dementia has not been fully elucidated yet. Inflammatory processes are hypothesized to play an important role as a driver for cognitive decline but the origin of inflammation is not clear. We hypothesize that disturbances in gut microbiome composition resulting in gut barrier dysfunction and bacterial translocation to systemic circulation and, further, the brain cause an inflammatory response and are associated with cognitive dysfunction in dementia.

Methods: To test this hypothesis, a cohort of 23 patients with dementia and 18 age and sex matched controls without cognitive impairments were studied. Gut microbiome composition, gut barrier dysfunction, bacterial translocation, and inflammation were assessed from stool and serum samples. Malnutrition was assessed by Mini Nutritional Assessment Short Form (MNA-SF), additionally, detailed information on drug use was collected. Microbiome composition was assessed by 16S sequencing, QIIME 2, and Calypso 7.14 tools.

Results: Dementia was associated with dysbiosis characterized by differences in beta diversity and changes in taxonomic composition. Potentially butyrate producing bacteria, including *Eubacterium rectale* and members of the *Lachnospiraceae* genus, were less abundant in demented individuals. Gut permeability was increased as evidenced by increased serum diamine oxidase levels, and systemic inflammation was confirmed by increased soluble cluster of differentiation 14 levels (sCD14). In a multivariate model, changes in microbiome composition of dementia patients could be explained by body mass index (BMI) and statin use. Notably, demented individuals took three times more prescription drugs than control, and three quarters of this group were malnourished.

Conclusion: Dementia is associated with changes in gut microbiome composition and increased biomarkers of gut permeability and inflammation. Demented individuals harbored less potentially butyrate producing bacteria, further promoting butyrate as a link between dysbiosis, gut barrier dysfunction, and cognitive decline. Moreover, malnutrition and drug intake were factors impacting microbiome composition. Taken together, increasing butyrate producing bacteria and targeting malnutrition may present promising therapeutic targets in dementia.

1 Introduction

1.1 *Alzheimer's disease (AD)*

Dementia - progressive cognitive impairment that impacts on activities of daily life - is currently estimated to affect 44 million people around the world. However, this number is anticipated to more than triple by 2050 since the population ages. The single leading cause of dementia is Alzheimer's disease (AD) comprising 50 to 70% of cases. First and foremost, AD is a condition of later life, approximately doubling in prevalence every five years after the age of sixty-five(1). However, 0.5% of cases develop symptoms earlier in life between ages 30 to 50, as those patients carry inheritable genetic mutations predisposing for so-called familial AD(2).

Episodic memory loss is the key symptom of AD, worsening over time of disease progression. Patients develop difficulties with tasks of daily living, especially planning and performing familiar processes, such as cooking or dressing. Severe disease state presents with aphasia, apathy, and impaired mobility. Patients die on average 8.5 years from beginning of symptoms(3,4).

AD is characterized by two cardinal neuropathological hallmarks, extracellular amyloid plaques (also called senile plaques) and intracellular neurofibrillary tangles (NFTs) that both spread across the brain, causing neurotoxic effects. Amyloid plaques are aggregations of amyloid β (Aβ), a peptide with either 40 or 42 amino acids (Aβ40 or Aβ42) processed by cleavage of the amyloid precursor protein (APP). NFTs, on the other side, are mainly composed of paired helical filaments built by hyperphosphorylated tau protein(5). Reaching a plateau still early in disease progression(6), amyloid burden does not correlate well with AD symptoms or neuron loss. Contrarily to that, the distribution pattern of NFTs reflects cognitive dysfunction as well as synaptic and neuronal degeneration(5). Eventually, the downstream consequence of synapse and neuron loss consists of macroscopic atrophy of brain matter, particularly of the limbic system, neocortical regions, and basal forebrain(5).

1.2 *Pathogenesis*

In familial AD, disease outbreak can be ascribed to inherited mutations in genes that encode for APP or its cleavage enzymes, resulting in overshooting production of senile plaques(2). On the contrary, sporadic AD is commonly regarded as a non-inheritable disease with unknown origin. However, this assumption is deceptive since 70% of disease risk can be attributed to genetic alterations(3). The single biggest genetic risk factor of sporadic AD is a variation in the Apolipoprotein E (APOE) gene encoding for apolipoprotein E, namely the variant ε4. Heterozygotic occurrence triples the odds to develop AD, which increase to even twelve-fold in homozygotes(7). Besides that, other serious risk factors include mid-life hypertension, diabetes mellitus (DM)(8), high cardiovascular risk(3), and obesity, although the latter has recently been questioned(9).

Nevertheless, while these genetic risk variants may facilitate the development of sporadic AD, they do not give answers to two fundamental questions: First, what induces the formation of amyloid plaques and NFTs in sporadic AD, and second, how do those lead to neurodegeneration and memory loss? Decades of research on the pathogenesis of AD have resulted in numerous hypotheses addressing these two questions. However, consensus on one explicit cause of disease has not been found so far.

1.3 How amyloid plaques and neurofibrillary tangles lead to neurodegeneration

1.3.1 Amyloid plaques

Amyloid plaques form as soon as the equilibrium between two separate cleavage pathways of APP becomes unbalanced. In physiological state, 90% of APP is cleaved over the so-called non-amyloidogenic pathway in comparison to only 10% over the amyloidogenic one. In AD, though, the balance is shifted towards increased amyloidogenic cleavage, resulting in overshooting production of Aβ that aggregates into plaques(10).

Essentially, the difference between amyloidogenic and non-amyloidogenic pathway boils down to down to the first cleavage step. In the non-amyloidogenic pathway, the enzyme α-secretase divides APP into a soluble (sAPPα) and a membrane-based part (α-C-terminal fragment [α-CTF] or also called C83). By contrast, in the amyloidogenic pathway, a different enzyme called β-secretase (beta-site APP-cleaving enzyme 1 [BACE1]) cuts APP into a shorter soluble (sAPPβ) and hence a longer membrane-based part (β-CTF or C99). Thus, the membrane-based peptides in both pathways are further separated by γ-secretase, leaving the APP intracellular domain (AICD) and P3 peptide as well as AICD and Aβ in non-amyloidogenic and amyloidogenic pathway, respectively(11) (Fig. 1).

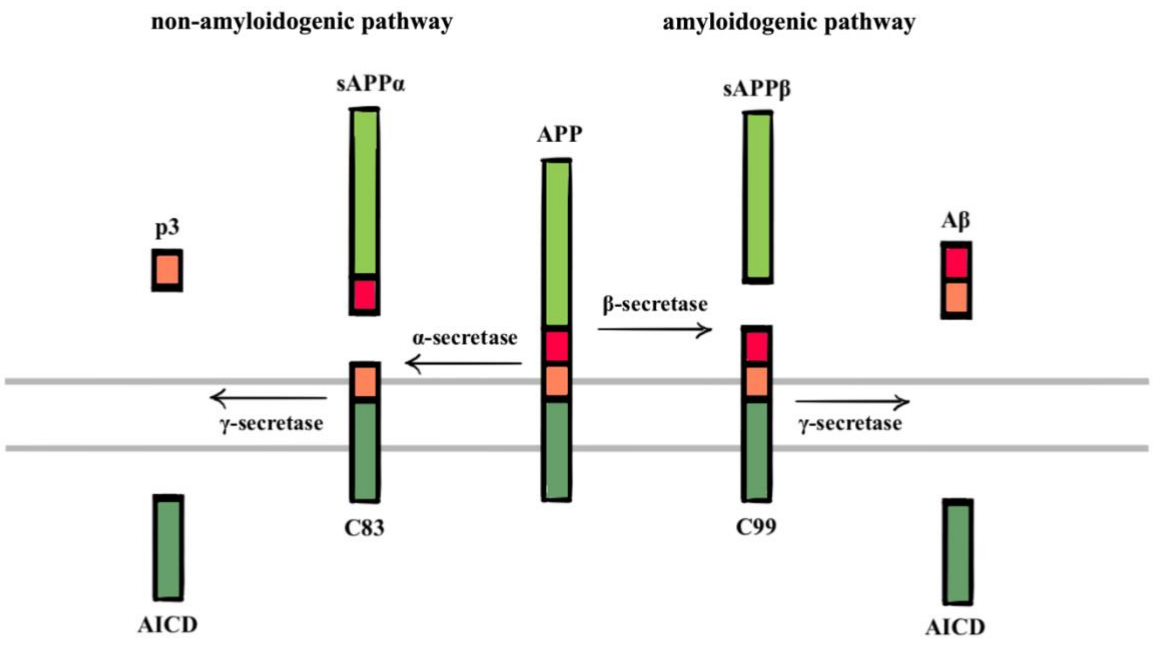

Figure 1: Amyloidogenic and non-amyloidogenic cleavage of amyloid precursor protein (APP). Abbreviations: Aβ = amyloid β, AICD = APP intracellular domain, sAPP = soluble APP, C83 = α-C-terminal fragment, C99 = β-C-terminal fragment, p3 = p3 protein. Adapted from: (12).

Before diving into the hypotheses what may induce this shift towards amyloidogenic cleavage, it is crucial to understand its result: how overshooting Aβ production elicits neurodegeneration.

On one hand, some APP cleavage products are crucial for normal nervous system function, including sAPPα being involved in synaptic plasticity, especially long term potentiation(13–16). On the other hand, Aβ increased over the normal range tends to aggregate and form oligomers, which again merge to protofibrils, fibrils and finally plaques. Aggregations of Aβ peptide are thought to harm neurons and glia cells far more than soluble Aβ alone(11). Not only elevated Aβ levels alone but rather an increased ratio of Aβ42/40 proved to be of great importance, since Aβ42 is much more prone to aggregation than Aβ40(17). Also, the C-terminal fragments are considered to exercise even greater neurotoxicity than Aβ alone(18).

All in all, a shift from non-amyloidogenic to amyloidogenic cleavage may induce various harmful effects on nerve cells, from diminished neuroprotective APP cleavage products to harmfully increased Aβ aggregation and CTFs(10).

1.3.2 Tau pathology

While accumulation of APP cleavage products induces axonal and synaptic defects, it also triggers aggregation of tau proteins in nerve cells, thereby setting off a cascade of even greater harm(19). Neuroscientist Rudy Tanzi from Harvard Medical School matches Aβ to starting “bush fires” of tau tangles, which in turn lead to a “forest fire” of neuroinflammation(20).

Regulated by phosphorylation, tau in its physiologic stage promotes microtubule polymerization and stabilization. In AD, hyperphosphorylated tau fails to bind microtubules, thereby inducing impairment of microtubule regulation. Instead, aberrant tau aggregates into NFTs, if formed in neuronal cell bodies, and neuropil threads, if built in axons or dendrites. Additionally, those tau fibrils act like seeds to convert physiologic tau to an abnormal state in a prion-like mechanism. Thus, tau pathology is initiated in a small number of brain cells from where it spreads to other regions(10).

1.3.3 Neurodegeneration through impaired neurotrophic signalling

Hyperphosphorylated tau protein miss-stabilizes filamentous actin, eliciting damage to the cytoskeletal system and impairing mitochondrial integrity as well as axonal signalling(10). Mitochondrial dysfunction, in turn, increases oxidative stress, spinning off a vicious cycle of destruction and further formation of amyloidogenic peptides and NFTs(21).

As pathologic tau deteriorates the sophisticated transport machinery that allows signal molecules, cell organelles, and trophic factors to travel along the axon, it simultaneously impairs the transport of the so-called nerve growth factor (NGF), a neurotrophic factor crucial for the survival of basal forebrain cholinergic neurons (BFCNs)(22–26). These neurons’ cell bodies are grouped in several nuclei of the basal forebrain, whereas their axons innervate the hippocampus, parahippocampus, entorhinal cortex, neocortex, and amygdala(27) - brain regions greatly involved in memory and attention. The selective loss of BFCNs and consecutive depletion of cholinergic signal transduction to their target cells mark disease progression in AD(28,29). Thus, a lack of supply with NGF may be the disease-defining step in AD pathogenesis.

In physiological state, BFCNs are provided with NGF by their target cells (i.e. hippocampus, cortex, etc.) to induce trophic effects in BFCNs and intensify synaptic cohesion as well as cholinergic signalling(23,26). Incorporated in an endosome, NGF is retrogradely transported to the nerve soma and further in the nucleus, where it encourages the expression and activity of choline acetyltransferase, an enzyme producing the BFCNs' specific neurotransmitter acetylcholine(30–32). To close the cycle, acetylcholine in turn travels back to the axon's presynaptic membrane, from where it is released into the presynaptic cleft upon excitation, enhancing signal transduction to those brain areas responsible for memory.

In this context, dysregulation of axonal transport via tau pathology results in a disruption of NGF transport to the soma and, further, loss of hippocampal and cortical innervation by BCFNs. This impairment in NGF transport is further enhanced as several APP cleavage products are implicated in the disruption of endocytosis and signalling endosome trafficking(33–38). Moreover, several AD risk variants comprise genes, whose proteins are involved in endocytosis and transport regulation(39).

All in all, this cascade originating in the imbalance in APP cleavage runs from hyperphosphorylation of tau protein to impairment of NGF trafficking along the axon and consequential degeneration of BFCNs failing to innervate memory areas in the brain.

1.3.4 Origins of amyloid plaque formation

The fundamental question that arises now is what initially disturbs the APP cleavage system. Dominating the field for over 25 years, yet the amyloid cascade hypothesis could not find a solution for this issue. Instead, its fundamental basis was unsettled as, first, Aβ plaques were also found in non-demented elderly(40,41), and second, trials focusing on depleting Aβ peptide failed at restoring memory deficits(20,42). Notably, increased occurrence of infections among the trial participants was observed in many anti-Aβ drug trials, especially meningoencephalitis(43–46), skin(46–50) as well as respiratory infections(46,48,49,51). Notably, APP is expressed in multiple tissues affected by those infections(52). The observation of increased risk for infections in Aβ-depleted individuals led to the conclusion that Aβ may somehow obtain an immunodefensive function.

Indeed, bacterial and viral deoxyribonucleic acid (DNA) were identified within Aβ plaques in brains of AD patients(53–55), which leaves the impression of Aβ entrapping pathogens to prevent them from spreading through the body. This theory, first introduced in 2002 by Robinson and Bishop as the "bioflocculant hypothesis"(56,57), was fortified by findings that Aβ production could be stimulated by infectious pathogens(58–64) and confirmed at the earliest in 2010 by direct evidence that Aβ exerted antimicrobial function against various gram-negative and -positive bacteria(64–66), viruses(63,67–69), and fungi(64–66). Aβ acts as an innate immune defence molecule by forming fibrils that surround and entrap pathogens(64) (Fig. 2) and possibly also by inserting cation channels in cell membranes, which disrupt intracellular ion homeostasis and lead to cell death(46,70,71). Production and secretion of Aβ is performed primarily by neurons yet also by most other cell types(72), especially by microglia(73) and, under inflammatory conditions, monocytes(74), both capable of eventually phagocytizing the entrapped invaders.

Further support of this theory is provided by the fact that brains of AD patients contain an increased pathogen load compared to control. Next generation sequencing revealed five to ten times more bacterial reads in AD brains than in non-AD brains(75). This finding is supported by other reports of increased cerebral abundance of bacteria such as *Chlamydia pneumoniae*(55), the periodontal species *Porphyromonas gingivalis*(76), and spirochetes, including periodontal *Treponemas* and *Borrelia burgdorferi*(77), in AD patients. A meta-analysis acknowledged significant associations between AD and bacterial infections, i.e., a ten-fold rise in occurrence of AD with spirochetal infection as well as a four-fold increase when infection with *C. pneumoniae* was detectable(78). Also, fungal infections were highly prevalent in brains and cerebrospinal fluid of AD patients, whereas none were observed in control(79–82). Additionally, higher risk of developing AD aligned with increased evidence of infection with Herpes simplex virus-1 (HSV-1) especially in the presence of the APOE ε4 allele (83–85). Besides, HSV-1 DNA was found in approximately 90% of Aβ plaques(54). HSV-6 and -7 were increased in AD post-mortem brains compared to control too. Notably, HSV-6 interacts with several modulators of the APP metabolism(86). Some studies also suggest associations between higher AD risk and infection with Human immunodeficiency virus (HIV)(87), *Helicobacter pylori*(88), *Toxoplasma gondii*(89), and Hepatitis B virus(90).

This evidence points towards a strong implication of pathogenic infection in AD. After a critical threshold may be surpassed, rates of pathogen-provoked Aβ aggregation may exceed the capacity for Aβ clearance, resulting in plaque formation and toxic effects on neurons and glia(91).

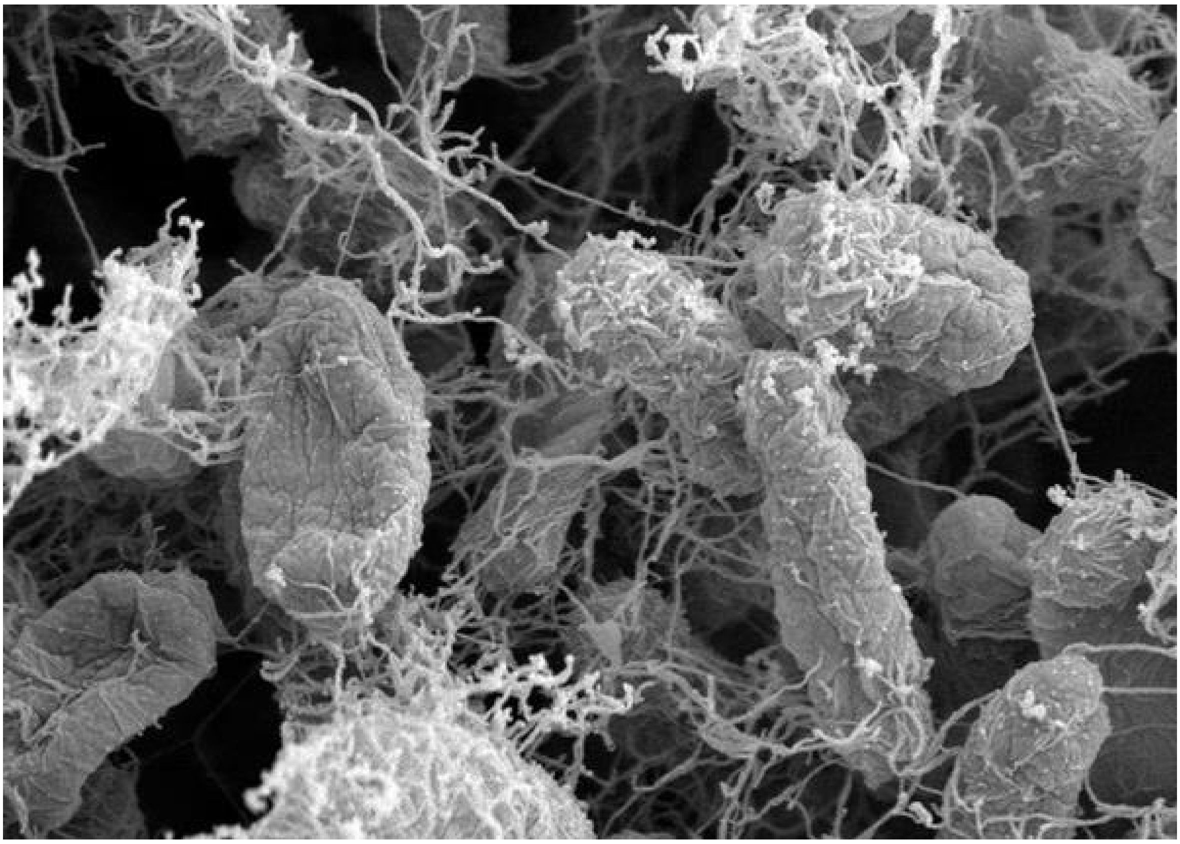

Figure 2: Pseudomonas aeruginosa entrapped by a mesh of amyloid-β fibrils. Credit: Deepak Kumar Vijaya Kumar. Source: (20). Permission for reproduction of the figure granted by Springer Nature on 14.10.2022.

1.4 Origin of microbes in AD brains

The increased rates of pathogenic particles in brain tissues of AD patients raise the question why and how those microbes invade the brain in the first place. For some microbes originating from the oral and nasal cavity, evidence was found that they enter the brain and cause Aβ plaque formation in AD.

In one study, mice were orally infected with *P. gingivalis*, a periodontal pathogen, that subsequently colonized the mice's brains and led to Aβ production(92). Bacteria causing periodontal disease may elicit damage to the local mucosa, thereby enhancing its permeability and enabling oral microbes to enter the blood stream. Once translocated to systemic circulation, bacteria may invade the brain and additionally trigger a systemic pro-inflammatory response, which would in turn reinforce neuroinflammation(93). In line with that, periodontal disease correlated significantly with presence of AD in a meta-analysis(94). However, some bacteria may not only reach the brain via systemic circulation but rather through direct travel along the trigeminal nerve. The periodontal disease-associated bacterium *Treponema* was found in trigeminal ganglia, pons, hippocampus, and frontal cortex, with greater concentrations and higher numbers of species in AD brains compared to control(95).

Furthermore, microbes present in the brain may as well originate from the nasal microbiome and travel directly via the olfactory nerve to the brain. The olfactory bulb is one of the earliest and hardest hit brain areas in AD pathogenesis and olfaction impairment was observed to be an early sign for AD(96). As there lies only a marginal distance between the nasal mucosa and the olfactory bulb as part of the brain, it is no wonder that post-mortem studies observed nasal microbes like *C. pneumoniae* located in the olfactory bulb of patients with AD(97). Moreover, *C. pneumoniae* was found in brain areas affected by AD among 89% of AD patients compared to only 5% of controls(98). Nevertheless, nasal microbiota may not only invade the brain via the olfactory system but also by infiltration of lung capillary monocytes that in turn cross the blood brain barrier (BBB)(99).

Another pathogen that may gain access to the central nervous system via the olfactory system is HSV-1. This virus was observed to migrate from olfactory epithelium to the brain via the olfactory nerve in a murine study(100) and to collocate with Aβ plaques in the brain(54). Notably, the presence of HSV-1 in the brain is associated with greater risk for AD especially with respect to carriers of the APOE ε4 allele(83,85,101).

Given that microbes from the oral and nasal microbiome were shown to invade the brain in AD, would it not be likely that the greatest microbial reservoir in the human body - the gut microbiome - may also yield bacteria that invade the brain in AD?

1.5 Gut microbiome involvement in AD

In order to act as catalysts in the development of AD, some may argue that intestinal bacteria would have to bridge a rather long distance between gut and brain. Yet, the microbiome and brain communicate with each other through a multifaceted, bidirectional signalling axis yielding multiple potential for disturbance of brain homeostasis.

This so-called “microbiota-gut-brain axis” integrates the immune system, vagal nerve and enteric nervous system, intestinal hormones, tryptophan metabolism, and microbial metabolites, including short-chain fatty acids, neurotransmitters, and neuroactive molecules. Even microbial particles themselves may invade the blood steam and engage in this signalling highway(102).

Gut dysbiosis – an increased number of pathogenic species or a pathological lack of species diversity within the gut(103) – spawns the extensive disruption of the microbiota-gut-brain axis. Consistent loss of homeostasis could eventually result in neurodegeneration.

Notably, the immune system plays an essential role in this axis since fundamental immunoeducation takes place in the intestinal mucosa. Via exposure of naïve immune cells to the microbial environment, the immune cells learn to differentiate between the body’s own versus foreign cells as well as between commensal versus pathogenic microbes(102).

In case of pathogenic gut colonization or invasion of pathogens into the body, the immune system reacts and elicits a systemic pro-inflammatory response battling against the infection but at the same time inflicting a systemic low-grade inflammation. This pro-inflammatory environment elicits damage to epithelial barriers, allowing for increased permeability and non-physiological particle exchange(102).

One of the bodies most crucial epithelial barriers, the BBB, is indeed more permeable in the presence of systemic inflammation(104). Additionally, BBB permeability is profoundly influenced by intestinal microbiota. Bred in the absence of microbes, germ-free (GF) mice exhibited increased BBB permeability together with reduced expression of tight junction proteins. However, exposure of GF mice to physiologic gut microbiota up-regulated tight junction proteins and normalized BBB permeability(105).

AD-associated neuroinflammation and dysbiosis combined with Aβ-deposition in cerebral vasculature cause the dysfunction and enhanced permeability of the BBB in AD. This BBB dysfunction impairs Aβ transport from the brain to peripheral circulation, thereby even accelerating cerebral Aβ aggregation(106,107).

Given the BBB disruption due to neuroinflammation and dysbiosis present in AD, it can be assumed that also the intestinal epithelial barrier is disturbed. A disrupted gut barrier would allow for bacteria to enter the blood stream, from where they can spread the body, trigger an even greater pro-inflammatory response, and finally, invade the brain across the impaired BBB. Inside the brain, amyloid peptide production would increase in order to entrap the translocated bacteria. As soon as this process abounds - which may as well take certain genetic predispositions - sporadic AD may be born.

So far, only few studies were carried out in order to explore the relationship between the gut microbiome and AD. Intestinal dysbiosis, especially a decrease in anti-inflammatory species, was evidenced in two mouse models of AD(108,109). Moreover, US researchers found a loss in species diversity and differences in taxa composition in stool samples from patients with mild to moderate AD(110). Recently, the disrupted microbiome of AD patients was proven to cause a dysregulation of the anti-inflammatory P-glycoprotein pathway(111). However, those studies did not include patients with severe AD, therefore no conclusions about the microbiome composition in advanced disease state can be drawn. Further, none of the studies above investigated potential factors leading to dysbiosis in AD, including gut barrier dysfunction and bacterial translocation or other important influences on the microbiome such as nutrition.

In our study, we wanted to show that, first, intestinal dysbiosis is present in patients with severe AD, and second, that gut barrier dysfunction in AD enables bacterial translocation to the blood stream and further the brain, representing the key factor in the development of AD.

2 Material and Methods

2.1 Study design and patient recruitment

In this prospective controlled cohort study, 23 patients with diagnosis of either Alzheimer's disease (n = 19) or mixed type dementia (Alzheimer type plus vascular type, n = 4) as well as 20 sex and age matched controls were recruited between July 2017 and March 2018 at the Albert Schweizer Hospital Graz and the University Hospital Graz, Austria. Inclusion criteria involved age of 18 years or older, diagnosis of Alzheimer or mixed type dementia made by a board-certified neurologist or psychiatrist according to ICD-10 (International Classification of Diseases 10) criteria, a Mini Mental State Examination (MMSE) Score equal to or less than 26, and written informed consent given by the patient or his legal representative. Patients with other forms of dementia, inflammatory bowel disease (IBD), liver cirrhosis, probiotic or antibiotic treatment within the last four weeks were excluded. Elderly controls enclosed men and women about the same age as the dementia patients, however definitely older than 18 years, not suffering from any known acute or chronic illness, and reaching an MMSE score greater than 26.
Based on their cognitive performance assessed by the Mini-Mental State Examination (MMSE), the study participants were allocated into dementia group (MMSE < 27) or control group (MMSE ≥ 27). According to this criterion, all dementia patients but also two participants originally recruited as controls matched into the criteria for the dementia group.

Calculation of sample size was carried out based on assumptions on species diversity measurements in healthy subjects (chao1 index of 14400 +/-2800) and its anticipated reduction in demented individuals (to chao1 index of 11500). With an alpha error of 0.05 and a beta error of 0.2, a sample size of 40 subjects was needed. As only one study visit was planned, dropouts were replaced one by one.

Routine blood chemistry analysis, including full blood count, renal function, electrolytes, liver function, albumin and total protein levels, and an inflammation parameter, were performed in each patient. Blood sampling for further analysis of biomarkers of intestinal permeability, bacterial translocation, and inflammation was only carried out in patients who could consent personally. Serum samples were taken after overnight fasting. Stool samples were collected for analysis of gut microbiome composition and markers of gut permeability by either the patient or a caregiver on the day or the evening before the study visit. Those samples were kept on 4°C until arrival at the hospital, where they were immediately frozen at -80°C. Further, physical examination, detailed documentation of medical history, including information about demographics, relevant former and current conditions, current medication, nutritional status, nutritional intake assessment, and dementia testing was performed.

The study (29–420 ex 16/17) was approved by the institutional review board Ethics Committee of the Medical University of Graz (IRB00002556) and has been registered at clinicaltrials.gov (NCT03167983) before its start. The study was performed according to the Declaration of Helsinki and Good Clinical Practise guidelines. Funds derived from the Center for Biomarker Research in Medicine.

2.2 Cognitive assessment

Cognitive function was quantified by two conventional tests for AD, the MMSE(112) and the clock-drawing test(113).

2.2.1 Mini-Mental State Examination

The MMSE(112) is a fast and suitable surrogate measure for the Clinical Dementia Rating (CDR), the standard tool used for grading the relative severity of dementia but limited by its length of administration(114,115). In order to establish a diagnosis of dementia, the MMSE adapted by Novartis (refer to Appendix) is used in conjunction with the patient's clinical history, a neurological examination, and other neuropsychological tests. Besides, it is frequently used to assess cognition in clinical studies on dementia(116).

Performed within five to ten minutes, the MMSE poses 30 questions or tests that aim to quantify the patient's abilities in orientation, object registration, attention, calculation, recall, language, and visual computation. A maximum of 30 points can be scored. According to the German S-3 guidelines on Dementia 2016, cognitive dysfunction in our cohort was classified as mild (MMSE score of 20-26), moderate (MMSE 10-19), and severe dementia (MMSE 0-9). MMSE scores of 27 to 30 were considered as no dementia(117).

2.2.2 Clock-drawing test

The clock-drawing test is a quick cognitive screening instrument that covers a wide range of abilities including executive functions. Further, it complements the MMSE by giving advantages in early detection of cognitive dysfunction and in monitoring mental decline(113). In this test, subjects are given a pre-drawn circle and asked to draw the numbers and afterwards the hands to show a specific time, usually ten minutes after eleven o'clock. Points are awarded for the correct placement of the number "12" (3 points), the inclusion of every number (2 points), two clock hands (2 points) and drawing the correct time (2 points). In total, 9 points can be scored. Zero to six points indicate the need of a thorough neurological examination, scores of seven points or higher do not require further investigation unless the examiner intends otherwise based on clinical appearance(118).

2.3 Malnutrition assessment

The Mini-Nutritional Assessment Short-Form (MNA-SF) was used to screen for malnutrition in our cohort. This 6-item test includes questions on decreased food intake, weight loss, body mass index (BMI), mobility, stress or acute disease, and neuropsychological problems. Individuals can score 0 to 14 points. A score of 12 or higher is considered normal, whereas values below 12 indicate malnutrition with a sensitivity of 97.9%, specificity of 100%, and diagnostic accuracy of 98.7%(119).

2.4 Assessment of gut permeability, bacterial translocation, and inflammation

Assessment of increased gut permeability is based on the understanding that substances within the gut lumen, such as bacterial endotoxins or products only enter systemic circulation after loss of intestinal barrier function. Also, enhanced levels of tight-junction barrier proteins can be measured in stool in the presence of gut barrier disruption.

Therefore, the tight-junction regulating protein zonulin as a surrogate parameter for tight-junction integrity was measured in stool samples of the study participants(120). Moreover, serum diamine oxidase (DAO) was quantified as another marker for intestinal barrier leakage, since damaged mucosal cells release DAO into circulation and high serum levels are tightly linked to gut barrier dysfunction(121).

Further, levels of bacterial products, including lipopolysaccharide (LPS, i.e. endotoxin), peptidoglycan, and bacterial DNA, were measured in the blood of the study participants. Also, indirect indicators of bacterial translocation were analysed in serum, namely lipopolysaccharide binding protein (LBP) and soluble (s)CD14, which is a marker for monocyte activation by LPS or other bacterial components(122).
As surrogate markers for systemic and intestinal inflammation, C-reactive protein (CRP) and faecal calprotectin were quantified, respectively. Elevation of this protein in faeces indicates migration of neutrophils to the gut mucosa, a process that occurs in intestinal inflammation(123).

Enzyme-linked immunosorbent assays (ELISA) were applied to quantify serum and faecal zonulin, faecal and serum calprotectin, serum DAO (Immundiagnostic AG, Bensheim, Germany), sCD14 (R&D Systems, Minneapolis, USA), and LBP (Hycult Biotech, Uden, The Netherlands), all according to the manufacturers' instructions. Reporter cells were used to detect endotoxin, peptidoglycan, and bacterial DNA in serum (HEK-Blue hTLR4, HEK-Blue hNOD2, and HEK-Blue hTLR9 cells, respectively) (Invivogen, Toulouse, France), as published previously(124).

2.5 Microbiome analysis

2.5.1 Preparation (DNA isolation and 16S-RNA sequencing)

Bacterial DNA was extracted from frozen stool samples through mechanical and enzymatic cell lysis using MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) (Roche, Mannheim, Germany) according to manufacturer's instructions. Since the hypervariable regions V1-V2 of the bacterial 16S ribosomal ribonucleic acid (rRNA) gene are highly specific for each species, they can be used for classification of DNA sequences to certain bacteria. Therefore, those DNA regions were amplified in a target-specific polymerase chain reaction (PCR) using the primers 27F and R357 (27F-AGAGTTTGATCCTGGCTCAG; R357-CTGCTGCCTYCCGTA) and sequenced with the Illumina MiSeq technique (Illumina, Eindhoven, The Netherlands). Sequencing was accomplished in collaboration with the Core Facility for Molecular Biology at the Center for Medical Research in Graz.

2.5.2 Analysis of microbiome composition and function

2.5.2.1 Pre-processing

FASTQ files (which are the output files of Illumina sequencing containing the bacterial DNA sequence, an identifier, and a quality score) were pre-processed using QIIME2(125) tools implemented in the user interface Galaxy (https://galaxy.medunigraz.at). After matching forward and reverse sequences and removing primer sequences, the data was denoised using DADA2(126). Denoising workflows remove redundant data and group highly similar sequences in order to avoid background noise through marginal sequencing errors in the later analysis. DADA2 involves quality filtering, removing chimeric sequences, merging paired-end reads (which are generated through sequencing of a DNA fragment starting from both ends(127)), dereplication (counting how often a sequence occurs and storing this information separately so that each sequence only has to be processed once), and a high resolution sample inference algorithm (reducing the genome set based on high sequence similarity). This process generates amplicon sequence variants (ASV), referred to as “features”, allowing for variation on an even finer scale than the formerly used “operational taxonomic units” (OTU, cluster of sequences that typically differ by less than 3%)(126). Features that occurred less than ten times in total and/or in less than two patients were excluded from analysis.

Taxonomy was assigned to the clustered sequences based on Silva 132 database release(128) at 99% OTU-level. Further classification was achieved through BLASTing of sequences in the NCBI database(129), a process that aligns and compares a sequence with a database of sequences via the computer algorithm BLAST (Basic Local Alignment Search Tool)(130). Chloroplasts and cyanobacteria were filtered from all samples to remove contaminants. The resulting mean sequencing depth after filtering was 41,631 (range 21,774–53,719) reads per sample.

2.5.2.2 Alpha diversity

For assessment of alpha diversity - the diversity of species within one sample - QIIME2 tools were used. First, data was rarefied with a sampling depth of 24,771 reads. This means that the read counts of all samples were reduced to this certain number by random selection of sequences, in order to not be misled by varying read counts when distinguishing variations in species number between samples. Alpha diversity was quantified by calculation of Chao1 index(131), Observed features, and Faith’s phylogenetic diversity (Faith PD)(132). Chao1 index estimates the count of unique features by extrapolating the number of rare species that may have been lost due to observation(131). By contrast, observed features directly counts the features in each sample. Faith PD considers the distance between two features on a phylogenetic tree(132).

2.5.2.3 Beta diversity

Beta diversity - the species diversity between samples - was analysed in QIIME2 using a rarefied feature table. Beta diversity was compared between groups based on calculation of Bray-Curtis dissimilarity(133), Jaccard distance(134), and UniFrac distance(135) metrics. Using different distance measures provides more insight into the source of microbiota variability(136).

Bray-Curtis dissimilarity is a quantitative measure that uses information on the species abundances of each sample to quantify the compositional dissimilarity between those samples. This dissimilarity metric is bound between 0 and 1, where 0 means that both samples share all the species and 1 that they have no species in common(133).

In contrast, the Jaccard index is a qualitative measure that does not take into account the absolute abundance of the species but rather relies on the presence or absence of species.

The UniFrac distance is a phylogenetic-based distance metric. It uses the evolutionary information of representative sequences to compare whether the samples differ in their microbial community in a particular evolutionary lineage. Concretely, it measures the phylogenetic distance between two taxa in a phylogenetic tree, represented by fractions of the tree's branch lengths. The unweighted UniFrac distance uses only the presence/absence of species on every branch, while ignoring the taxa abundance. Contrarily, the weighted UniFrac distance accounts for the species abundance, however, becoming less sensitive to the abundance changes on those branches with small proportions(136).

The resulting distance matrices for each distance metric were compared between groups using Principal Component Analysis (PCoA). PCoA identifies latent variables, so-called principal components (PC), that explain as much of the variation in the data as possible. The first two, strongest PCs are displayed as axis in a PCoA plot and samples are illustrated as data points distributed along those two axes. This representation allows for the visual identification of clusters of samples, e.g. clustering of diseased patients versus controls(137).

To test for significant clustering, Permutational Multivariate Analysis of Variance (PERMANOVA)(138) was used. PERMANOVA compares the total sum of squared dissimilarities among samples belonging to different groups to that of objects belonging to the same group. The magnitude of group separation is indicated by the pseudo-F value; however, the significance of the test is usually of more interest than the magnitude of pseudo-F(139).

To further evaluate beta diversity differences, Redundancy Analysis (RDA) was performed using the web-based software Calypso 7.14 (http://cgenome.net/calypso/)(140). RDA is a direct extension of multiple regression. While multiple regression models the effect of an explanatory matrix (including variables characterizing the samples, e.g., BMI or CRP) on one single response variable, RDA models the effect of this explanatory matrix on a response matrix (e.g., a matrix including the abundance of each species in each sample). The resulting plot resembles a PCoA plot allowing as well for the identification of clustering(141).

2.5.2.4 Taxon comparison

Taxon comparison was carried out in Calypso based on an unrarefied feature table containing relative species abundances, which were calculated through total sum scaling (TSS) and square root transformation. Differentially abundant taxa were detected with Analysis of Composition of Microbiomes (ANCOM)(142). This method accounts for the underlying structure of microbiome data thereby reducing false discovery rates while maintaining high statistical power in the discovery of differentially abundant taxa on an ecosystem level(142). ANCOM compares log-ratios of taxon abundances between samples, thus avoiding false discoveries due to shifts in relative abundances(143). The test infers differential abundance to a taxon based on how often the abundance of this taxon relative to other taxa is significantly different between the observed groups (W-value) (144). To deal with the problem that log transformations cannot be computed on taxa of zero abundance, ANCOM adds pseudo counts e.g. the constant 1 to all counts before calculation(143).

Moreover, the supervised machine learning algorithm Linear discriminant analysis Effect Size (LEfSe)(145) identified taxa that could be used to discriminate between groups. A cut-off value of Linear discriminant analysis (LDA) > 3.5 was used.

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)(146) estimated the metagenome content of the samples based on the identified bacteria. The resulting data was matched to the Kyoto Encyclopedia of Genes and Genomes

(KEGG)(147) in order to predict up- and downregulated bacterial functions (i.e. KEGG pathways) in disease state.

All analyses were carried out on feature, genus, family, class, order, and phylum level.

2.6 Other statistical analyses

All other statistical analyses were conducted in SPSS version 27.0 (SPSS Inc., Chicago, Illinois, USA) and R(148) version 4.1.3 (packages: “Hmisc”, “mice”, “ranger”, “vegan”, “faraway”)(149–153). Distribution of data was assessed by Shapiro-Wilk test. In case of normally distributed data, t-tests were chosen, whereas Mann-Whitney U tests were used for non-parameterised distributions. Categorical data was compared by Fisher’s exact test. Benjamini-Hochberg correction for multiple testing was performed to decrease the number of false discoveries (false discovery rate = FDR) (package “Hmisc”(149)). Strength and direction of monotone relationships between variables were determined using Spearman’s rank correlation coefficient. All statistical tests were 2-sided and p-values of less than 0.05 were considered statistically significant. Missing values were imputed by Multivariate Imputation by Chained Equations (package “mice”(150)) based on random forests (package “ranger”(151)). Univariate and multivariate RDA was performed to identify variables that explain the variance in microbiome composition (package “vegan”(152)). To account for collinearity between these potential explanatory variables, variables with correlation coefficients > 0.75 or with Variance inflation factors (VIF) > 2 (package “faraway”(153)) were excluded. The remaining explanatory variables were scaled for multivariate RDA (package “vegan”(152)).

All graphs illustrating results were created in R(148) version 4.1.3 by using the packages “ggplot2”(154), “ggpubr”(155), “ggConvexHull”(156), and “tidyverse”(157).

3 Results

3.1 Patient characteristics

Forty-four subjects were assessed for eligibility to participate in the study, of which one declined to participate, 25 patients were allocated to the dementia group and 18 to control. As for two dementia patients, neither blood nor stool samples could be gathered due to the patients' oblivion to collect their stool and inability to consent personally, for which reason they were excluded from analysis. The remaining patients were classified into different stages of disease severity according to MMSE score: 8 patients were categorised into mild dementia state, whereas 9 suffered from moderate and 6 from severe dementia. Of two patients with moderate dementia no stool samples could be collected due to the above-mentioned reasons, which is why they were included only in analysis of laboratory blood values but neither in stool biomarkers nor in microbiome analysis (Fig. 3).

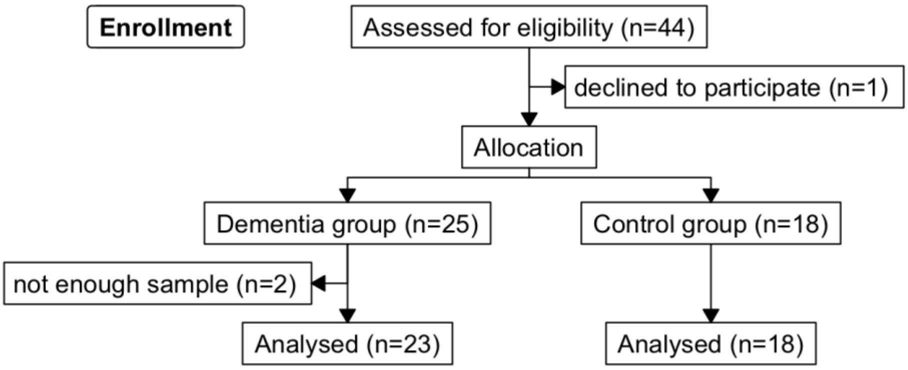

Figure 3: Patient flow chart

Dementia and control group did not differ in age or sex; however, the demented patients had a significantly lower BMI ($p = 0.012$) and worse nutritional status indicated by MNA-SF score ($p < 0.001$). In line with that, also serum albumin and total protein were significantly reduced in the dementia group ($p = 0.005$ and $p = 0.014$, respectively). Moreover, demented patients displayed significantly reduced erythrocyte count ($p = 0.018$) and haemoglobin levels ($p = 0.045$) compared to control. As expected, dementia patients achieved significantly lower scores in the MMSE and clock-drawing test ($p < 0.001$ for both). Whereas most control subjects scored nearly full points in both tests, demented patients achieved only a median of 16 out of 30 points in the MMSE and 3 out of 9 points in the clock-drawing test (Table 1).

Within the dementia group, MMSE showed significant positive correlations with albumin ($r_s = 0.707$, $p = 0.005$), clock-drawing test results ($r_s = 0.666$, $p = 0.005$), and erythrocyte count ($r_s = 0.669$, $p = 0.005$). Further, albumin correlated negatively with the number of prescribed drugs ($r_s = -0.635$, $p = 0.008$) and positively with erythrocyte count ($r_s = 0.624$, $p = 0.008$) and total protein ($r_s = 0.544$, $p = 0.034$) in demented patients. BMI did not correlate with MMSE or clock drawing test results.

Notably, dementia patients took three times more prescription drugs than controls ($p < 0.001$, Table 1). Anti-dementia drugs, antidepressants, sedatives, vitamin D, laxatives, antipsychotics, metamizole, and opioids were nearly exclusively prescribed to patients in the dementia group. No significant differences in drug intake between demented and control subjects could be found with antihypertensive drugs, non-steroidal anti-inflammatory drugs (NSAIDs), proton pump inhibitors (PPIs), thyroid hormones, statins, anticoagulation, calcium and magnesium supplements, phytotherapeutics, antidiabetics, and paracetamol (Table 2). Most drugs were prescribed as fixed medication, whereas metamizole, paracetamol, laxatives, and sedatives were prescribed as needed.

Table 1: Patient characteristics. Data are given as median and 95% confidence interval unless stated otherwise.

	Dementia patients (n=23)	**Controls (n=18)**	**p-value**
Age (years)*	78 (74;82)	75 (73;77)	n.s.
Gender (f/m) (n)	15/8	7/11	n.s.
BMI (kg/m2)*	24.6 (23.1; 26.1)	28.0 (25.6; 30.4)	p=0.012
MMSE	16 (13;21)	30 (30;30)	p<0.001
Clock drawing test	3 (0;5)	8 (7;9)	p<0.001
Number of prescription drugs*	9 (7;11)	4 (2;5)	p<0.001
MNA-SF	11 (11;13)	14 (14;14)	p<0.001
Leukocytes (10^9/L)	6.6 (6.2;8.3)	6.1 (5.5;7.5)	n.s.
Erythrocytes (10^{12}/L)*	4.3 (4.1;4.6)	4.8 (4.5; 5.0)	p=0.018
Thrombocytes (10^9/L)	220 (216;248)	216 (205;222)	n.s.
Haemoglobin g/dL*	13.0 (12.2;13.8)	14.1 (13.4;14.7)	0.045
Creatinine (mg/dL)	0.9 (0.8;1.0)	1.0 (0.9;1.1)	n.s.
Bilirubin (mg/dL)	0.6 (0.5;0.9)	0.6 (0.5;0.6)	n.s.

Albumin (g/dL)*	3.9 (3.7; 4.1)	4.2 (4.1;4.4)	p=0.005
Total protein (g/dL)	7.0 (6.8;7.3)	7.5 (7.3;7.6)	p=0.014
CRP (mg/l)	5 (3;11)	2 (1.2; 3.4)	n.s.

*indicates variables with normal distribution for which data are given as mean and 95% confidence interval. Abbreviations: BMI = body mass index, MMSE = Mini-Mental state examination, MNA-SF = Mini-Nutritional Assessment Short-Form, CRP = C reactive protein.

Table 2: Drug intake in dementia patients and controls.

	Dementia patients (n=23)	**Controls (n=18)**	**p-value**
Anti-dementia drugs (n)	13	0	<0.001
Antidepressants (n)	17	1	<0.001
Sedatives (n)	13	1	<0.001
Vitamin D (n)	16	1	<0.001
Laxatives (n)	10	0	0.002
Antipsychotics	8	0	0.006
Metamizole (n)	8	0	0.006
Opioids (n)	10	1	0.011
Antihypertensives (n)	**12**	**10**	**n.s.**
NSAIDs (n)	**9**	**6**	**n.s.**
PPIs (n)	**6**	**7**	**n.s.**
Thyroid hormones (n)	**8**	**3**	**n.s.**
Statins (n)	**3**	**6**	**n.s.**
Anticoagulation (n)	2	4	n.s.
Calcium and magnesium supplements (n)	4	2	n.s.
Phytotherapeutics (n)	4	2	n.s.
Antidiabetics (n)	3	1	n.s.
Paracetamol (n)	3	0	n.s.

Bold print: drug classes selected for further analysis due to distribution and frequency. Abbreviations: NSAIDs = non-steroidal anti-inflammatory drugs, PPIs = proton pump inhibitors.

3.2 *Differences in gut microbiome composition*

3.2.1 Alpha Diversity

Alpha diversity, as measured by Chao1 index, observed features, and Faith PD, did not differ between demented patients and controls (Fig. 4A-C). However, alpha diversity decreased stepwise with worsening disease stage, though this finding also did not reach significance using one-way ANOVA (chao1: F = 0.693, n.s.; observed features: F = 0.689, n.s.; Faith PD: F = 0.578, n.s.) (Fig. 4D-F). There were no significant correlations between alpha diversity metrics and MMSE or clock drawing scores.

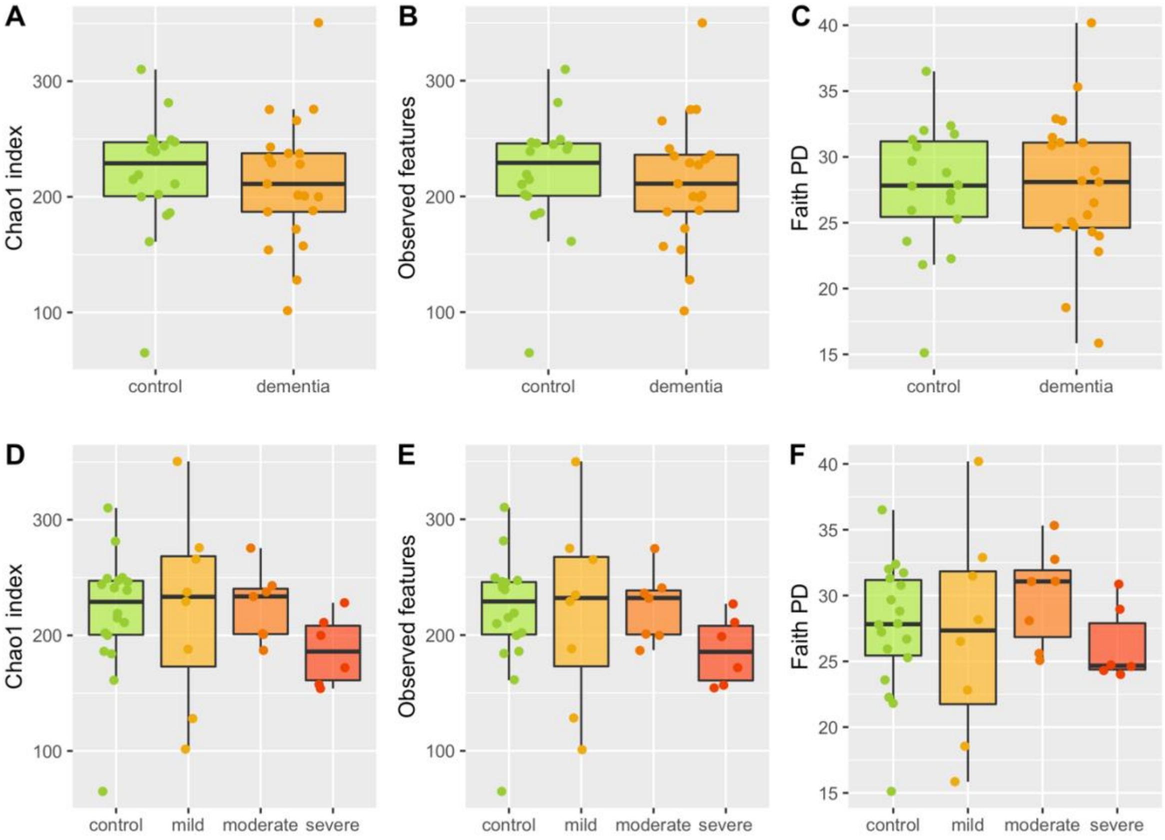

Figure 4: Differences in alpha diversity of stool microbiome between dementia patients and control. (A) Chao1 index, **(B)** observed features, and **(C)** Faith's phylogenetic diversity (Faith PD) between dementia patients and control (no significant differences). **(D)** Chao1 index, **(E)** observed features, and **(F)** Faith PD between controls and different stages of cognitive dysfunction (no significant differences).

3.2.2 Beta Diversity

PCoA based on Bray-Curtis dissimilarity showed clear clustering of dementia patients in contrast to controls (PERMANOVA: F=1.41, p=0.037) (Fig. 5A) and also clustering regarding dementia stages (F=1.27, p=0.021) (Fig. 5B, Table 3). These findings were strengthened by similar results based on other dissimilarity metrics including Jaccard and Unweighted UniFrac (Table 3). Moreover, significant clustering of both groups as well as of disease stages was also seen in RDA analysis (Variance 34.25, F=1.31, p=0.003 and Variance 94.71, F=1.22, p=0.001; respectively) (Fig. 5C and D).

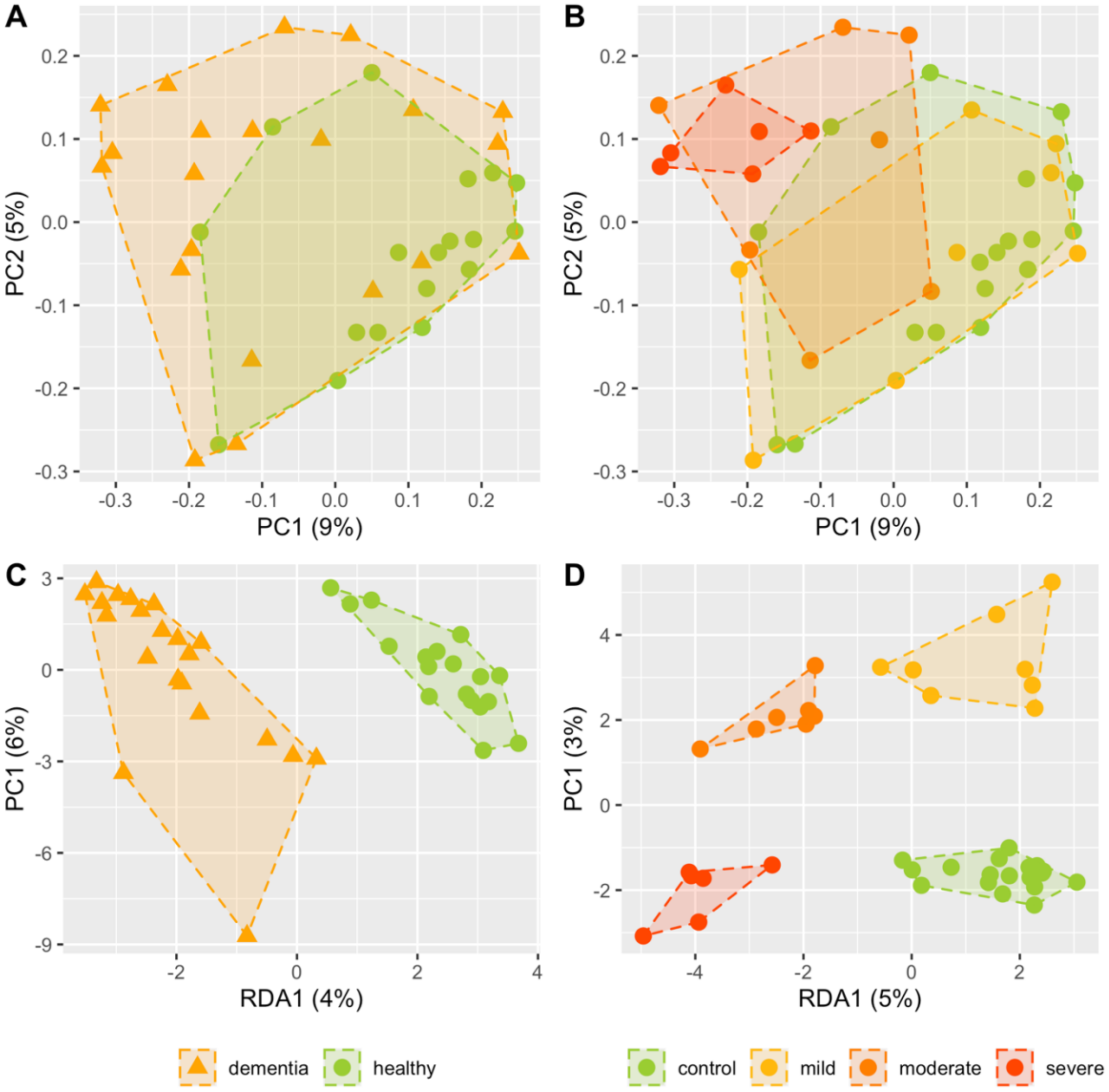

Figure 5: Differences in beta diversity between dementia patients and control. (A) Principal Component Analysis (PCoA) based on Bray-Curtis dissimilarity showed significantly higher between-group than within-group distances of dementia patients

compared to controls as well as **(B)** of different stages of cognitive dysfunction and controls. **(C)** Redundancy Analysis (RDA) showed significant clustering of dementia versus control group and **(D)** of different stages of cognitive dysfunction and controls. Abbreviation: PC = principal component.

Table 3: Comparison of beta diversity metrics between dementia group and control as well as between stages of cognitive dysfunction.

	Pseudo-F	**p-value**
Dementia versus control		
Bray-Curtis	1.41	0.037
Jaccard	1.51	0.021
Unweighted UniFrac	1.64	n.s.
Weighted UniFrac	0.91	n.s.
Stages of cognitive dysfunction		
Bray-Curtis	1.27	0.021
Jaccard	1.34	0.021
Unweighted UniFrac	1.61	0.024
Weighted UniFrac	0.95	n.s.

The given distance matrices were compared using Permutational Multivariate Analysis of Variance (PERMANOVA).

3.2.2.1 Taxon-specific analysis

LEfSe identified one family, 5 genera, and 7 features to differ between demented patients and control (Fig. 6A). ANCOM confirmed that three taxa, namely the feature *[Eubacterium] rectale*, an uncultured *Lachnospiraceae* species, and the genus *Lachnospiraceae bacterium NK4A136 group* were less abundant in stool of demented than control subjects (Fig. 6B).

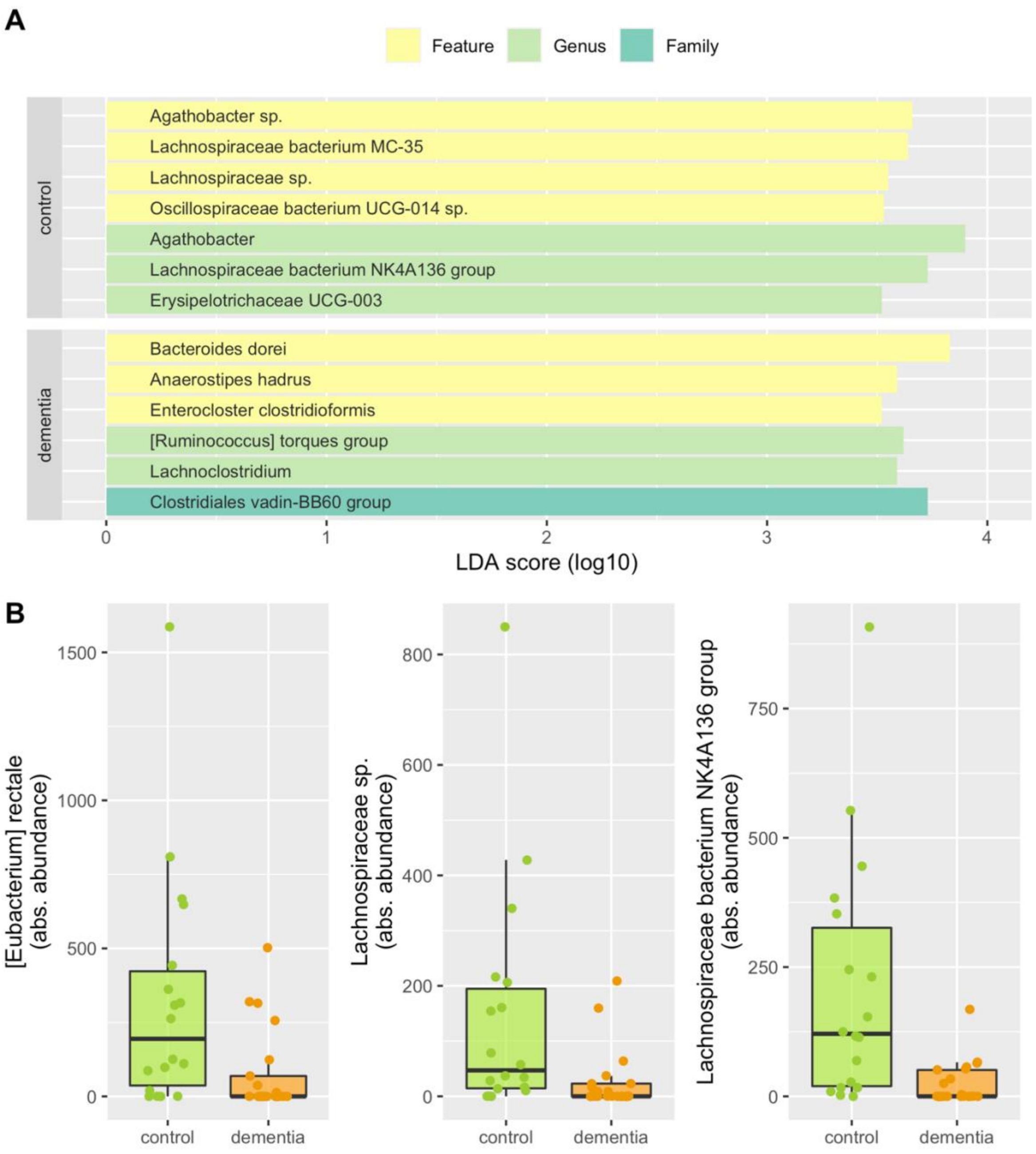

Figure 6: Taxon-specific differences between dementia patients and controls. (A) Features selected by Linear discriminant analysis Effect Size (LEfSe) to discriminate between dementia patients and controls. **(B)** Differentially abundant taxa between dementia and controls identified by ANCOM. Square brackets around a genus indicate that the name awaits appropriate action by the research community to be transferred to another genus. Abbreviations: ANCOM = Analysis of Composition of Microbiomes, abs. = absolute, LDA = Linear discriminant analysis, sp. = species.

When looking at different disease stages of dementia, LEfSe identified one class, 3 orders, 3 families, 18 genera, and 20 features to significantly differ in abundance between stages (Fig. 7A). Of those, the feature *Enterocloster clostridioformis* (formerly *Clostridium clostridioforme*) and the genus *Eisenbergiella* were also discriminated by ANCOM to be increased in severe disease stage. ANCOM also identified the family *Lachnobacillaceae* as being most abundant in moderate dementia (Fig. 7B).

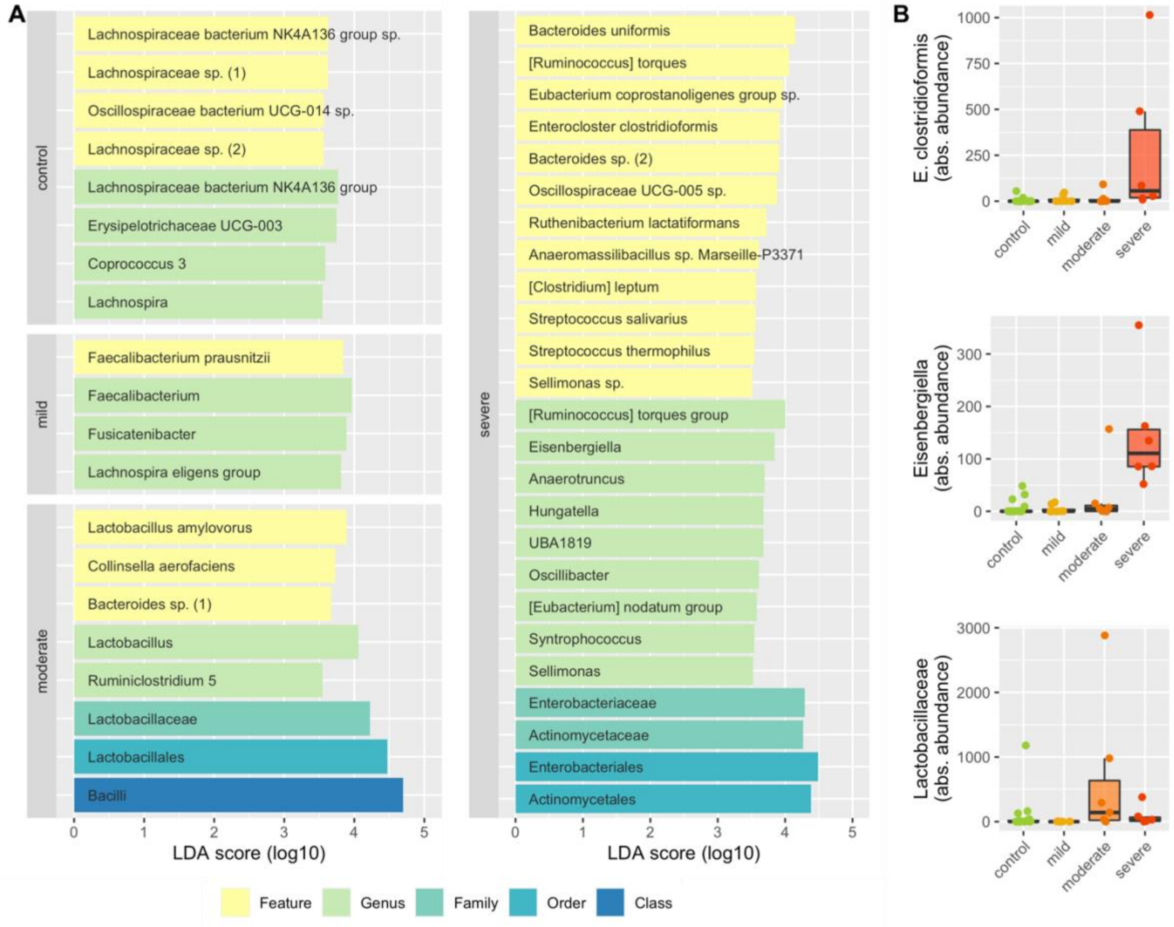

Figure 7: Taxon-specific differences between different stages of cognitive dysfunction and controls. (A) Features selected by Linear discriminant analysis Effect Size (LEfSe) to discriminate between dementia different stages of cognitive dysfunction and controls. **(B)** Differentially abundant taxa between stages of cognitive dysfunction identified by ANCOM. Square brackets around a genus indicate that the name awaits appropriate action by the research community to be transferred to another genus. Abbreviations: ANCOM = Analysis of Composition of Microbiomes, abs. = absolute, *E. clostridioformis* = *Enterocloster clostridioformis,* LDA = Linear discriminant analysis, sp. = species.

3.3 Differences in gut microbiome function

PICRUSt as a predictor of metagenome function showed no differences in functional alpha diversity (chao1, observed richness) or beta diversity (Bray-Curtis, Jaccard) neither between dementia and control group nor in comparison between different stages of cognitive dysfunction.

LEfSe identified 9 functions to be discriminatory between demented individuals and controls, including functions involved in bacterial energy metabolism and protein processing (Fig. 8A). ANCOM did not find any functions differing significantly between both groups.

When looking at different stages of cognitive dysfunction, additional metabolic functions and further toxin degradation functions were differentially abundant between dementia stages according to LEfSe (Fig. 8B). Additionally, ANCOM found that the functions “drug metabolism - cytochrome P450” and “electron transfer carriers” to raise in abundance in moderate and severe dementia, whereas the function “polycyclic aromatic hydrocarbon degradation” diminished with increasing disease severity (Fig. 8C).

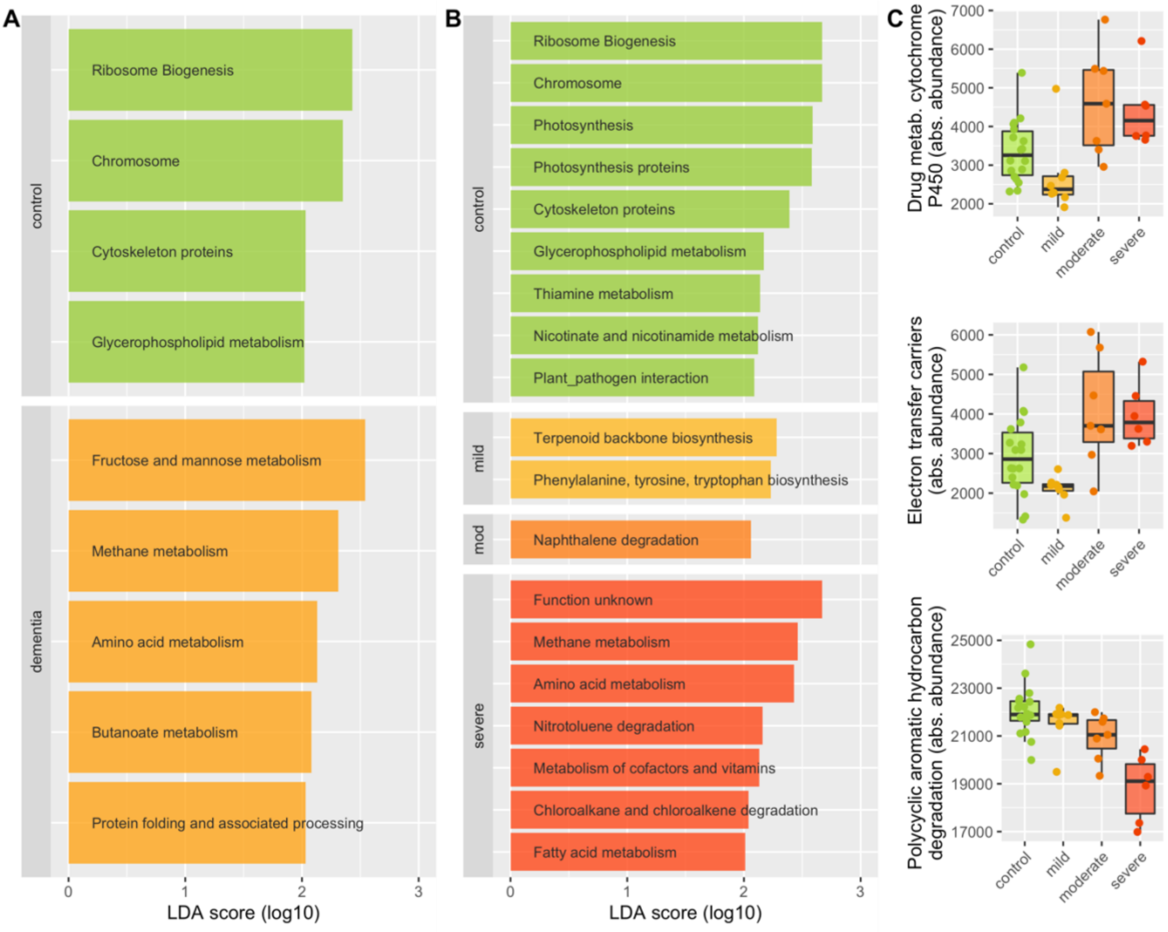

Figure 8: Differences in predicted metagenome function. Bacterial functions selected by Linear discriminant analysis Effect Size (LEfSe) to discriminate between **(A)** dementia and control group and **(B)** different stages of cognitive dysfunction. **(C)** Differentially abundant functions between stages of cognitive dysfunction identified by ANCOM. Abbreviations: ANCOM = Analysis of Composition of Microbiomes, LDA = Linear discriminant analysis, mod = moderate, metab. = metabolism, abs. = absolute, sp. = species.

3.4 Gut barrier dysfunction, inflammation, and bacterial translocation

Dementia patients had two times higher serum DAO and increased sCD14 levels, suggesting a link to gut barrier dysfunction and increased inflammation due to bacterial translocation in dementia. However, other markers for increased gut permeability or bacterial translocation as well as all tested biomarkers for inflammation remained insignificantly different between groups (Table 4). Also, MMSE and clock-drawing levels did not correlate with any of the tested biomarkers.

Table 4: Biomarker for gut barrier dysfunction, inflammation, and bacterial translocation. Data are shown as median and 95% confidence interval.

	Dementia patients (n=23)	Controls (n=18)	p-value
Serum diaminooxidase (U/ml)	20.8 (9.7;29)	11.2 (8.4; 13.8)	0.025
Faecal zonulin (ng/ml)	33.8 (31.2; 57)	55.1 (40.8; 76.7)	n.s.
Faecal calprotectin (ng/ml)	31.5 (26.6; 85.8)	49.0 (18.2; 66.3)	n.s.
C-reactive protein (mg/L)	5 (4; 11)	2 (1.2;3.4)	n.s.
Serum lipopolysaccharide binding protein (µg/ml)	17.9 (16.1; 18.6)	20.0 (14.6; 21.3)	n.s.
Soluble CD 14 (µg/ml)	2.4 (1.9; 3.1)	1.8 (1.7; 2.1)	0.022
Endotoxin (EU/ml)	0.26 (0.0; 0.33)	0.25 (0.09; 0.53)	n.s.
Peptidoglycan* (ng/mL)	0.96 (0.26; 1.66)	0.42 (0.30;1.05)	n.s.
Bacterial DNA (µM)	0.06 (0.00;1.46)	0.7 (0.0; 1.29)	n.s.

*peptidoglycan was only measurable in 12% of the samples, therefore median and confidence interval only for the positive samples are shown. Abbreviations: CD = cluster of differentiation, EU = endotoxin units.

3.5 *Influence of medication on gut microbiome composition and function*

As demented patients took three times more prescription drugs than controls, we wanted to further investigate the impact of medication on the microbiome and its possible role in the development of dementia. We therefore analysed the usage of drugs equally prescribed in both groups for a possible influence on microbiome composition and function. These included PPIs, antihypertensive drugs, statins, NSAIDs, and thyroid hormones. As paracetamol, antidiabetics, calcium and magnesium supplements, anticoagulants, and phytotherapeutics were taken by less than 15% of our study cohort, they were not included into the analysis.

3.5.1 Influence of medication on alpha and beta diversity

None of the drugs impacted on alpha diversity (Chao1, observed features, Faith PD) and only statin use had a significant influence on beta diversity indicated by PCoA

(PERMANOVA: Jaccard: F=1.35, p=0.032) and RDA (Variance 35.58, F=1.36, p=0.003). No other drug influenced beta diversity metrics.

Table 5: Comparison of beta diversity metrics between medication users and non-users.

		PPIs	**Statins**	**Antihyper-tensives**	**NSAIDs**	**Thyroid hormones**
Bray-Curtis	**(Pseudo-) F**	0.82	1.31	0.96	0.99	1.13
	p-value	n.s.	n.s.	n.s.	n.s.	n.s.
Jaccard	**(Pseudo-) F**	1.05	1.35	1.10	1.09	0.99
	p-value	n.s.	**0.032**	n.s.	n.s.	n.s.
Unweighted UniFrac	**(Pseudo-) F**	0.87	1.48	1.65	1.29	0.85
	p-value	n.s.	n.s.	n.s.	n.s.	n.s.
Weighted UniFrac	**(Pseudo-) F**	0.96	1.36	1.76	0.81	0.70
	p-value	n.s.	n.s.	n.s.	n.s.	n.s.
RDA	**Variance**	26.47	35.58	27.61	29.00	25.12
	F	1.01	1.36	1.05	1.10	0.95
	p-value	n.s.	**0.003**	n.s.	n.s.	n.s.

The given distance matrices were compared using Permutational Multivariate Analysis of Variance (PERMANOVA). Redundancy analysis (RDA) results are displayed in the last row. Abbreviations: NSAIDs = non-steroidal anti-inflammatory drugs, PPIs = proton pump inhibitors.

3.5.2 Impact of medication on taxon abundance

3.5.2.1 Proton-pump inhibitors (PPIs)

LEfSe selected one order, three families, five genera, and two features to discriminate between PPI users and non-users. Out of those taxa, ANCOM identified the families *Streptococcaceae* and *Barnesiellaceae* and the normally oral-based feature *Streptococcus salivarius* to be more abundant in stool of PPI users, whereas the genus *Holdemanella* decreased with PPI use (Fig. 9A).

3.5.2.2 Antihypertensive drugs

As to antihypertensive drugs, LEfSe identified the feature *Ruminoclostridium 6 species (sp.)*, the genus *Oscillospiraceae NK4A214* (both belonging to the family *Oscillospiraceae* and the order *Eubacteriales*), one unclassified genus as well as family, and the family *Prevotellaceae* (order *Bacteroidales*) to increase with its usage. ANCOM confirmed the importance of the genus *Oscillospiraceae NK4A214 group* and the unclassified family, while adding the butyrate-producing feature *Anaerobutyricum hallii group sp.* (order *Eubacteriales*) to the list of taxa increased by antihypertensive drugs. In terms of taxa reduced by use of antihypertensives, LEfSe discriminated the genera *Blautia* and *Clostridium innocuum group* (both belonging to the order *Eubacteriales*), the genus *Bacteroides* and its corresponding family *Bacteroidaceae*, order *Bacteroidales*, class *Bacteroidia*, and phylum *Bacteroidetes*. Both LEfSe and ANCOM identified the family *Lactobacillaceae* to also decrease with antihypertensive use (Fig. 9B).

3.5.2.3 Statins

LEfSe identified the phylum *Firmicutes*, two families out of the order *Eubacteriales* (phylum *Firmicutes*), 6 genera, and 7 features to increase with statin use. Out of those taxa, ANCOM confirmed the raised abundance of the phylum *Firmicutes* as well as the feature *Faecalibacterium prausnitzii* in statin users. However, the phylum *Actinobacteria* and its corresponding classes *Actinobacteria* and *Coriobacteria*, the order *Enterobacteriales* (phylum *Proteobacteria*), two families, two genera, and one feature decreased with statin use according to LEfSe. ANCOM verified the genus *Erysipelatoclostridium*, the family Enterobacteriaceae and corresponding order Enterobacteriales, as well as the phylum *Actinobacteria* and corresponding genus *Coriobacteria* to be less abundant in statin users (Fig. 9C).

3.5.2.4 Non-steroidal anti-inflammatory drugs (NSAIDs)

According to LEfSe, NSAID use was associated with increased abundances of the feature *Bacteroides sp*., the feature *Anaerostipes hadrus* and its corresponding genus *Anaerostipes* (belonging to the order *Eubacteriales*), two other genera named *Terrisporobacter* and *Intestinimonas* and the family *Peptococcaceae* (all three also belonging to *Eubacteriales*), as well as one unclassified family, the order *Iziplasmatales* (phylum Bacilli), and the class

Mollicutes and its corresponding phylum *Tenericutes*. ANCOM verified the feature *Bacteroides sp.* as well as the genus *Anaerostipes* to be increased in stool of NSAID users (Fig. 9D).

ANCOM also found 27 orders and 19 classes to distinguish between NSAID users and non-users. However, this is likely due to the fact that ANCOM anticipates a bimodal distribution of resulting W-values (indicating significance), one for significant and one for insignificant taxa. If the results are unimodally distributed, there is a 50% chance for the results to be significant or not. Therefore, a great number of significant taxa in ANCOM should be treated with caution, which is why we excluded the results from further consideration.

3.5.2.5 Thyroid hormones

Thyroid hormone use increased four features including two *Bacteroides* species, *Enterocloster clostridioformis*, and *Ruminococcus 1 sp*. as well as the genus *Blautia*, of which the latter three belong to the order *Eubacteriales*. Moreover, the genera *Parasutterella, Lactococcus,* and *Akkermansia*, and the latter's corresponding family (*Akkermansiaceae*), order (*Verrucomicrobiales*), class (*Verrucomicrobiae*), and phylum (*Verrucomicrobia*) were more abundant in thyroid hormone users. ANCOM confirmed the rise of the feature *Bacteroides sp.* and genus *Lactococcus* associated with thyroid hormone use (Fig. 9E).

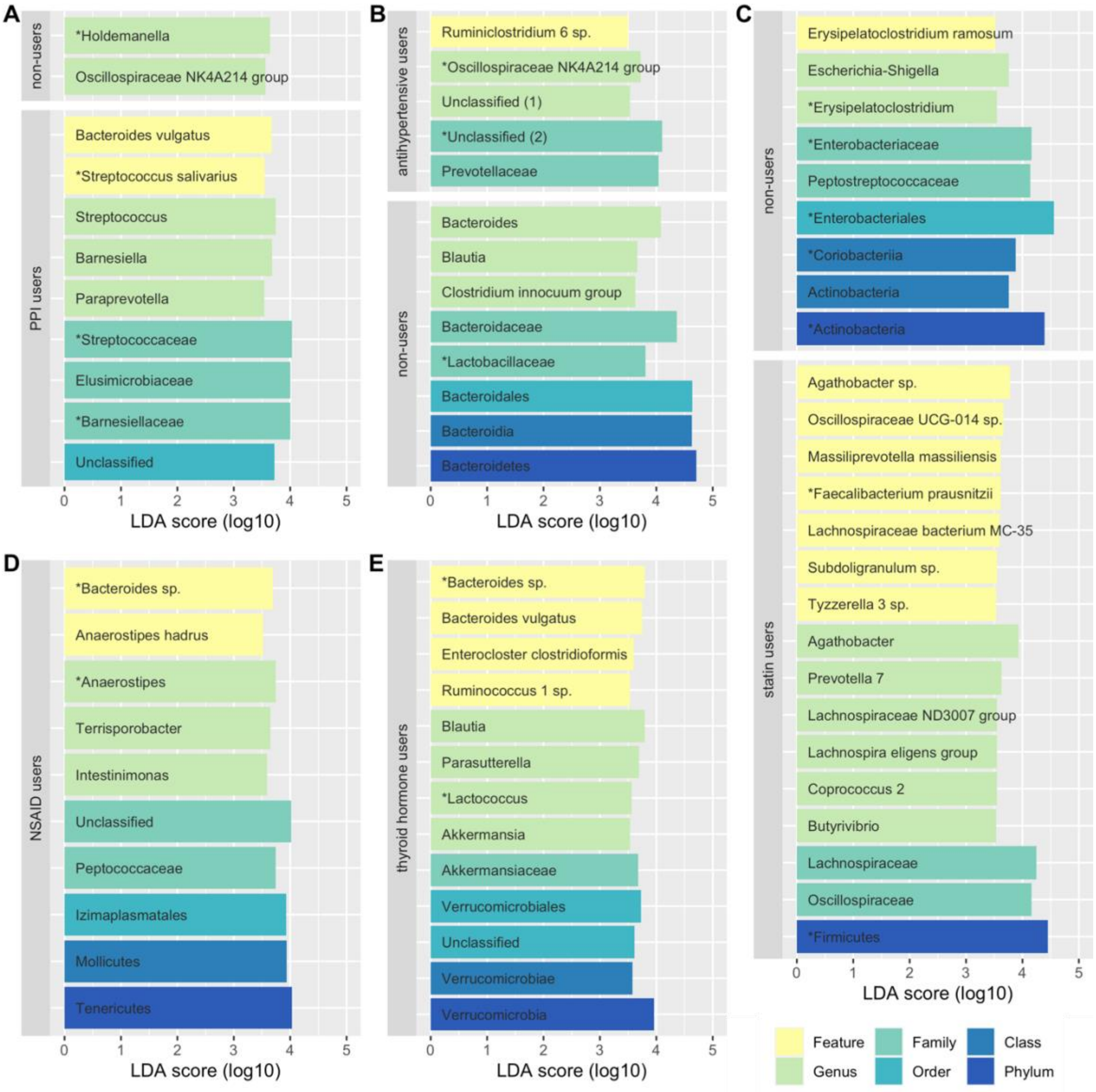

Figure 9: Influence of medication on microbiome composition. Features selected by Linear discriminant analysis Effect Size (LEfSe) to discriminate between **(A)** PPI users and non-users, **(B)** antihypertensive drug users and non-users, **(C)** statin users and non-users, **(D)** NSAID users and non-users, and **(E)** thyroid hormone users and non-users. *indicates taxa also found by ANCOM to discriminate between NSAID users and non-users. Abbreviations: ANCOM = Analysis of Composition of Microbiomes, LDA = Linear discriminant analysis, NSAID = non-steroidal anti-inflammatory drug, PPI = proton pump inhibitor, sp. = species.

3.5.3 Influence of medication on microbiome function

The intake of prescribed medication also influenced various microbiome functions. PICRUSt did not show any differences in functional alpha or beta diversity associated to drug use. However, LEfSe selected many functions altered by drug intake, which are listed in Fig. 10A. Additionally, ANCOM identified decreased alpha-linolenic acid metabolism in the gut microbiome of statin users, increased ether lipid metabolism associated with intake of antihypertensives, and raised steroid biosynthesis in stool of thyroid hormone users (Fig. 10B). Also, two functions named „bladder cancer“ and „Chagas disease American trypanosomiasis“ were associated with statin use according to ANCOM, though these results might represent further errors due to the mechanism PICRUSt uses to predict metagenome function.

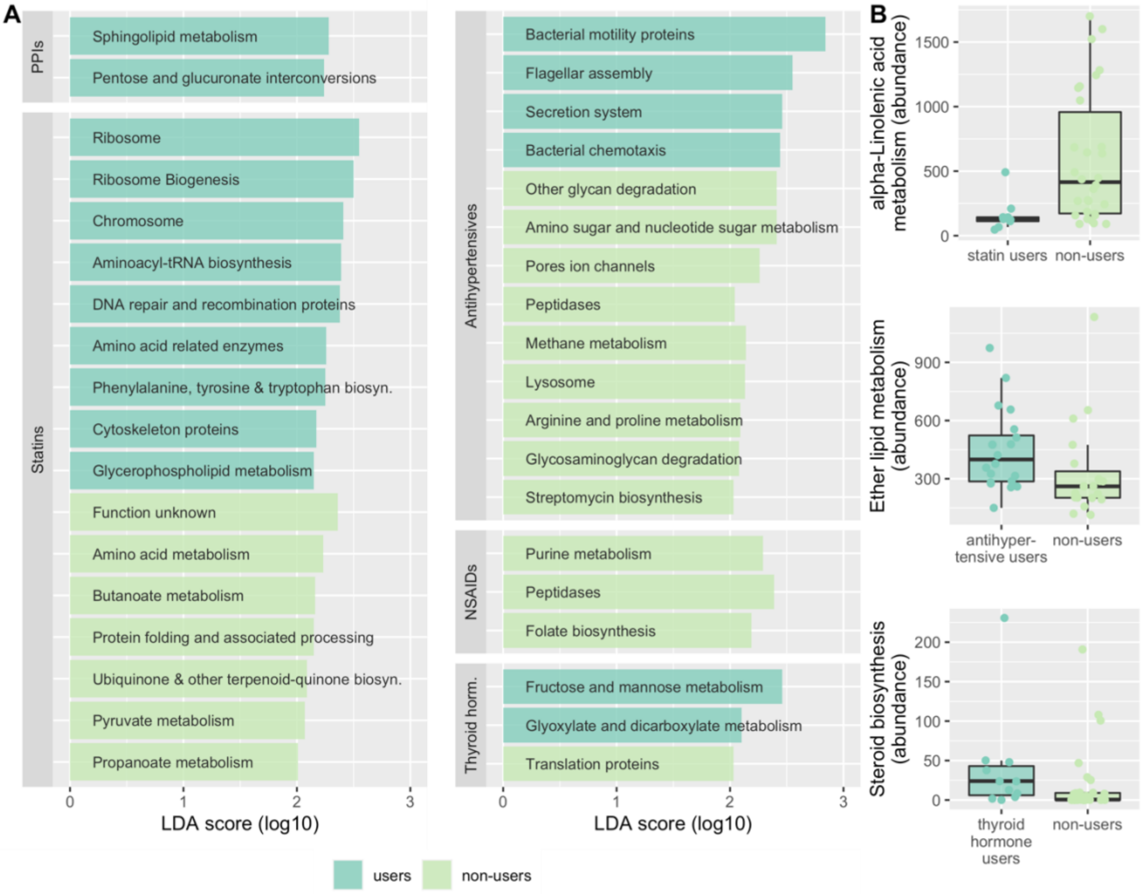

Figure 10: Influence of medication on predicted metagenome function. (A) Bacterial functions selected by Linear discriminant analysis Effect Size (LEfSe) to discriminate between drug users and non-users. **(B)** Differentially abundant functions between drug users and non-users. Abbreviations: biosyn. = biosynthesis, horm. = hormones, LDA = Linear discriminant analysis, NSAID = non-steroidal anti-inflammatory drug, PPI = proton pump inhibitor.

3.5.4 Impact of medication on gut inflammation

We wanted to investigate if drug use influences gut permeability, inflammation, or bacterial translocation, and found significant positive associations between PPI use and faecal calprotectin levels (PPI use: 92.5 ng/ml (50.2; 120.5); PPI non-use: 28.1 ng/ml (20.8; 47.9); p=0.008) as well as between antihypertensive drug use and increased CRP levels (antihypertensive use: 6 mg/dl (3; 11); antihypertensive non-use 1.3 mg/dl (1;4); p=0.016).

3.6 Influence of nutrition on gut microbiome composition and function

Malnutrition was present in 74% in demented individuals according to MNA-SF, while none of the controls was malnourished. Therefore, microbiome composition in malnourished subjects was very similar to that found in demented patients. Aligning with the observations in the dementia group, malnutrition also did not influence alpha diversity (chao1, observed features, Faith PD), but had an impact on beta diversity according to RDA (Variance 32.31, F=1.24, p=0.013). PCoA results based on distance matrices indicated a distinct difference in Jaccard index, which, however, did not sustain FDR correction.

Table 6: Comparison of beta diversity metrics between malnutrition and control.

	Pseudo-F	p-value
Malnutrition versus control		
Bray-Curtis	0.256	n.s.
Jaccard	0.011	n.s.
Unweighted UniFrac	0.092	n.s.
Weighted UniFrac	0.412	n.s.

The given distance matrices were compared using Permutational Multivariate Analysis of Variance (PERMANOVA).

Both LEfSe and ANCOM selected the feature *Oscillospiraceae UCG-014 sp.* and the genus *Lachnospiraceae bacterium NK4A136 group* to be associated with a healthy nutritional state. Moreover, LEfSe also identified the corresponding genus *Oscillospiraceae UCG-014* to be increased in good nutritional condition, whereas the genus *Anaerobutyricum halli group* was more abundant in malnourished patients. When comparing dementia versus control group, the feature and genus *Oscillospiraceae UCG-014* as well as the genus *Lachnospriaceae bacterium NK4A136 group* were all also diminished in demented individuals (Fig. 11A and B).

In terms of microbiome function, LEfSe indicated that pyruvate metabolism was enhanced in malnourished patients (LDA score = 2.04) and ANCOM identified atrazine and styrene degradation to decrease in this group.

Concerning the influence of malnutrition on gut barrier dysfunction, bacterial translocation, and inflammation, lower MNA-SF values were significantly correlated to higher sCD14 levels (r_s = -0.508, p = 0.027). However, no differences in the tested biomarkers were detected between the malnourished and the control group.

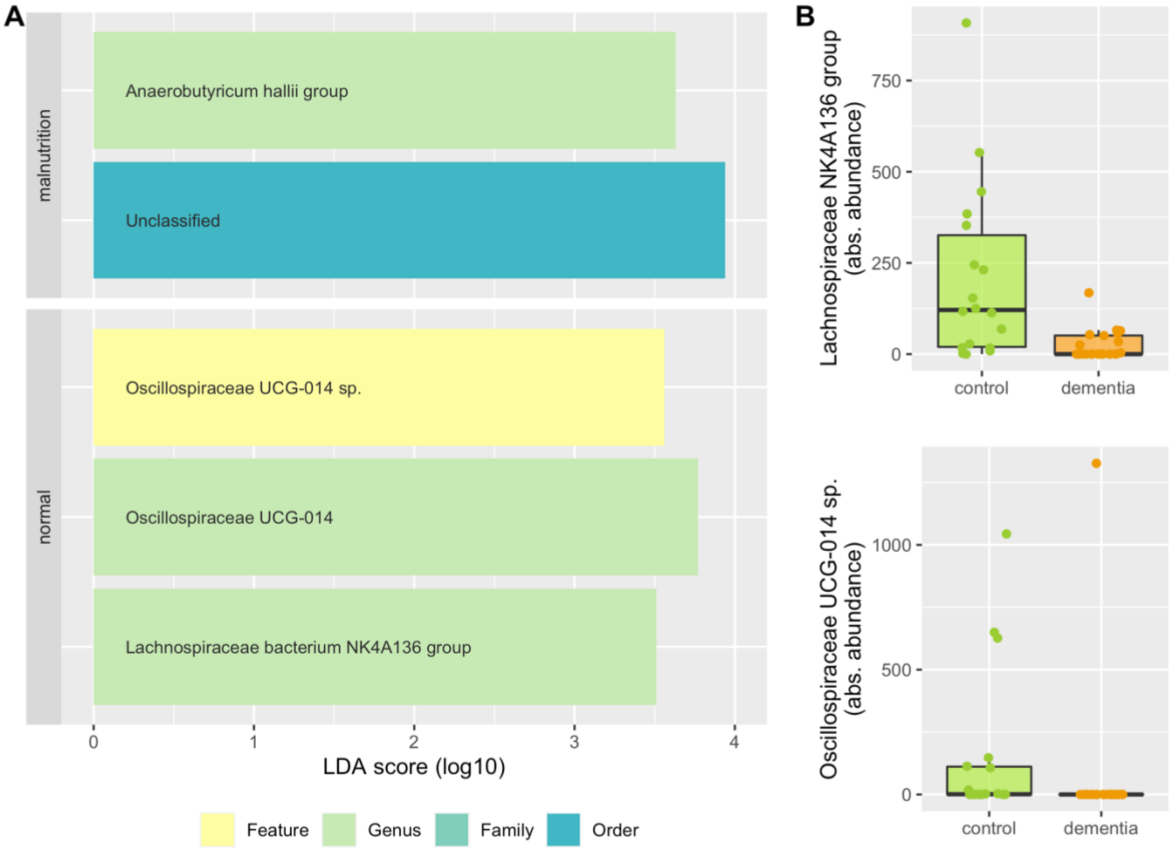

Figure 11: **Taxon-specific differences between malnourished patients and control. (A)** Features selected by Linear discriminant analysis Effect Size (LEfSe) to discriminate between malnourished patients and participants with normal nutritional status. **(B)** Differentially abundant taxa between malnourished and non-malnourished participants. Abbreviations: LDA = Linear discriminant analysis, abs. = absolute, sp. = species.

3.7 Multivariate Redundancy Analysis

To identify the main drivers of dysbiosis in dementia, we assessed the correlation of clinical variables and biomarkers with microbiome composition by performing univariate and multivariate RDA.

Univariate RDA indicated that the following parameters were associated with changes in the gut microbiome of dementia patients versus controls and between stages of disease severity ($p<0.1$): BMI, albumin, total protein, soluble CD14, statin use, NSAID use, MNA-SF, MMSE, clock-drawing test levels, age, sex, and number of prescribed drugs (Table 7).

Table 7: Redundancy analysis (RDA) with explanatory variables of microbiome composition changes.

Explanatory variable	Dementia vs. controls	Severity of dementia
BMI	Variance = 32.85 F = 1.26 **p = 0.014**	Variance = 32.85 F = 1.26 **p = 0.009**
Albumin	Variance = 42.41 F = 1.64 **p = 0.001**	Variance = 42.41 F = 1.64 **p = 0.001**
Total protein	Variance = 32.44 F = 1.24 **p = 0.016**	Variance = 32.44 F = 1.24 **p = 0.01**
Soluble CD14	Variance = 36.36 F = 1.4 **p = 0.001**	Variance = 36.36 F = 1.4 **p = 0.001**
DAO	Variance = 27.8 F = 1.06 p = 0.196	Variance = 27.8 F = 1.06 p = 0.201
PPIs	Variance = 26.47 F = 1.01 p = 0.417	Variance = 26.47 F = 1.01 p = 0.398
Statins	Variance = 35.58 F = 1.36 **p = 0.002**	Variance = 35.58 F = 1.36 **p = 0.001**
Antihypertensives	Variance = 27.61 F = 1.05 p = 0.158	Variance = 27.61 F = 1.05 p = 0.205
NSAIDs	Variance = 29 F = 1.1 **p = 0.086**	Variance = 29 F = 1.1 **p = 0.093**
Thyroid hormones	Variance = 25.12 F = 0.95 p = 0.721	Variance = 25.12 F = 0.95 p = 0.711
Zonulin	Variance = 28.03 F = 1.07 p = 0.202	Variance = 28.03 F = 1.07 p = 0.219

Calprotectin	Variance = 23.29 F = 0.88 p = 0.848	Variance = 23.29 F = 0.88 p = 0.857
Endotoxin	Variance = 20.5 F = 0.77 p = 0.987	Variance = 20.5 F = 0.77 p = 0.986
LBP	Variance = 25.53 F = 0.97 p = 0.632	Variance = 25.53 F = 0.97 p = 0.625
Peptidoglycane	Variance = 23.54 F = 0.89 p = 0.868	Variance = 23.54 F = 0.89 p = 0.866
Bacterial DNA	Variance = 24.96 F = 0.95 p = 0.671	Variance = 24.96 F = 0.95 p = 0.697
MNA-SF	Variance = 36.27 F = 1.39 **p = 0.002**	Variance = 36.27 F = 1.39 **p = 0.001**
MMSE	Variance = 44.25 F = 1.71 **p = 0.001**	Variance = 44.25 F = 1.71 **p = 0.001**
Clock-drawing test	Variance = 36.26 F = 1.39 **p = 0.005**	Variance = 36.26 F = 1.39 **p = 0.001**
Age	Variance = 29.86 F = 1.14 p = 0.062	Variance = 29.86 F = 1.14 p = 0.075
Sex	Variance = 36.06 F = 1.38 **p = 0.001**	Variance = 36.06 F = 1.38 **p = 0.003**
Number of drugs	Variance = 34.81 F = 1.34 **p = 0.004**	Variance = 34.81 F = 1.34 **p = 0.002**
CRP	Variance = 24.38 F = 0.92 p = 0.747	Variance = 24.38 F = 0.92 p = 0.72

Abbreviations: BMI = body mass index, DAO = diamino oxidase, PPIs = proton pump inhibitors, NSAIDs = non-steroidal anti-inflammatory drugs, LBP = lipopolysaccharide binding protein, MNA-SF = Mini Nutritional Assessment Short Form, MMSE = mini mental state examination, CRP = C-reactive protein.

Collinearity analysis was performed to identify variables strongly associated to each other, which thus could interfere with multiple analysis. VIF below 2 were shown for BMI, albumin, MMSE, clock-drawing test, and MNA-SF.

The final multivariate RDA model included only explanatory variables with $p<0.1$ in the univariate analysis and excluded variables with VIF > 2 in collinearity analysis. In the multivariate RDA model, BMI and statin use remained as the only explanatory variables for microbiome changes between dementia and control patients as well as between stages of cognitive dysfunction (Table 8).

Table 8: Multivariate Redundancy Analysis (RDA) to identify the most important explanatory variables for microbiome composition changes.

Explanatory variable	**Dementia vs. control**	**Severity of dementia**
BMI	Variance = 33.18 F = 1.29 **p = 0.006**	Variance = 33.18 F = 1.29 **p = 0.008**
Total protein	Variance = 29.19 F = 1.14 p = 0.067	Variance = 29.19 F = 1.14 p = 0.070
Soluble CD14	Variance = 29.06 F = 1.13 p = 0.072	Variance = 29.06 F = 1.13 p = 0.079
Statins	Variance = 32.06 F = 1.25 **p = 0.009**	Variance = 32.06 F = 1.25 **p = 0.014**
Clock-drawing test	Variance = 25.89 F = 1.01 p = 0.376	Variance = 25.89 F = 1.01 p = 0.374
Age	Variance = 25.88 F = 1.01 p = 0.427	Variance = 25.88 F = 1.01 p = 0.409
Sex	Variance = 27.63 F = 1.08 p = 0.137	Variance = 27.63 F = 1.08 p = 0.154
NSAIDs	Variance = 28.17 F = 1.10 p = 0.098	Variance = 28.17 F = 1.10 p = 0.107

Abbreviations: BMI = body mass index, NSAIDs = non-steroidal anti-inflammatory drugs.

4 Discussion

In this cross-sectional pilot study, distinct changes in the gut microbiome composition of AD patients compared to control were observed. These compositional changes imply the loss of butyrate-producing bacteria in the development of AD and consecutive destruction of the intestinal epithelial barrier, indicated by an increase in markers for gut permeability and inflammation. Our findings therefore align with the concept of a disrupted gut-brain-axis implied in the development of dementia. Further, malnutrition and prescription drug use were identified as the main drivers for the observed dysbiosis in AD.

4.1 Alpha diversity

Against intuitional anticipations, alpha diversity did not differ between AD patients and controls in our cohort. Merely, a tendency towards decreasing alpha diversity with worse MMSE scores was noticed.

Comparing literature, information on gut alpha diversity in human AD studies is relatively scarce. Vogt et al. reported reduced alpha diversity in AD patients with mainly mild to moderate severity(110). In patients with mild cognitive impairment, a clinical picture preceding AD, alpha diversity was unchanged(158). Other gut microbiome studies in human did not look at alpha diversity.

Regarding AD animal models, though, evidence on the variety of microbial species in AD is diverging. One study in AD rats reported decreased alpha diversity(159), whereas two models of AD mice(109,160) as well as mice transplanted with faeces from AD patients(161) exhibited increased richness. Also, there is conflicting evidence whether diversity is rising or falling with increased age of the animals(108,160,162). Three AD mouse studies did not observe any differences in alpha diversity between AD and control microbiomes(163–165).

The discrepancies in alpha diversity between our study and other human AD studies may be explained by differences in the selection of controls, as microbiome composition varies considerably between individuals(166). This variety even exceeds in old age(167), as elderly people are repeatedly exposed to community-altering events such as intake of medication, polypharmacy, infections, concomitant antibiotic use, and hospital stays. Therefore, choosing elderly controls means accepting a non-healthy control and a greater variance within the control cohort itself. This variance could account for the exhibited deviation in findings for alpha diversity.

Further, differences in sequencing techniques may rationalize diverging alpha diversity results(168), as a lower sequencing depth will withhold the presence of low-abundant species and thereby mask a possible gap in alpha diversity between patients and controls. However, the mean sequencing depth in our study was 41,631 (range 21,774–53,719) reads per sample and the rarefaction curves of most samples reached a plateau, indicating that the sequencing depth was appropriate for analyses and should therefore not interfere with alpha diversity measurements(169). Another possible explanation for the missing significance of our alpha diversity findings could be the low number of patients in each disease state of dementia, resulting in low statistical power.

In general, decreased diversity is a commonly accepted phenomenon of pathologic state, indicative of an unbalanced microenvironmental composition, in which few pathologic species have outnumbered the physiologic microbiome. However, more and more evidence emerges, suggesting that this principal may not be generally applicable for all diseases accompanied by dysbiosis. Studies on major depressive disorder and autism, for example, report increased alpha diversity associated with pathology(170,171). Therefore, the tendency towards either rising or falling alpha diversity may vary in meaning from disease to disease. Yet, more research must be carried out in order to answer compellingly if and how alpha diversity is altered in AD.

4.2 Clustering of AD patients

In our study, we could show that the AD cohort clearly clustered apart from the controls. This indicates the presence of one or several factors that make the microbial composition of AD patients distinguishable from normal state, i.e., the rise or fall of certain bacterial taxa. Moreover, the different stages of AD severity clustered separately as well, implying that microbiome composition constantly changes over the course of disease. Given this proof of existing change, it was imperative to detect those shifts on every taxonomic level and, further, to find explanations for their cause and effect.

4.3 Alterations on phylum level

In our study cohort, no significant shifts from healthy to AD could be detected in any phylum. However, most AD-associated variations occurred within the phyla Firmicutes and Bacteroidetes. These are the two main intestinal phyla, together representing 90% of all bacteria(172). The ratio between them underlies considerable variations due to factors such as age(173), location(174), nutrition, or physical activity(175). Due to this high degree of variation, it is difficult to connect shifts of the Firmicutes:Bacteroidetes ratio with human diseases, yet efforts in this direction are being made(176).

Two other studies on microbiome composition in AD patients described disease-associated shifts between these two phyla. In the first study, Vogt et al. observed a decrease in Firmicutes and increase in Bacteroidetes abundance in US American AD patients(110), as already anticipated from the results of several studies in AD model mice(108,109,160). In contrast, the second study, carried out in a Chinese cohort, reported a stable Firmicutes abundance and a mild but significant decrease in Bacteroidetes associated with AD(177). The latter findings are supported by a study showing a slight yet insignificant tendency towards higher Firmicutes and lower Bacteroidetes abundances in US patients with mild cognitive impairment than in controls (158).

In our study, however, some taxa within the phylum Firmicutes increased, while others decreased, resulting in no net alteration on phylum level. Of all bacteria belonging to the phylum Bacteroidetes, we could solely observe increases of several *Bacteroides* species in moderate to severe dementia, but no rises or falls in any other taxa of the phylum Bateroidetes. Raised proportions of the genus *Bacteroides* in AD patients were also found by Haran et al. and Vogt et al.(110,111).

4.4 Bacteroidetes: Rise in Bacteroides species

Members of the genus *Bacteroides* display widespread characteristics, ranging from facultative pathogenic to anti-inflammatory and immunomodulatory.

Bacteroides fragilis exemplifies this phenomenon. Like all Bacteroidetes, *B. fragilis* is a gram-negative bacterium and expresses LPS on its outer membrane. High levels of *B. fragilis*, thus, provoke greater LPS influx into the blood stream, as proven in GF mice colonised with human intestinal microbiota(178). Fittingly, the abundance of this pathogen was shown to be raised in patients with AD(111). In cognitively impaired elderly, however, its abundance was lower than in control(179). Still, *B. fragilis* is not only considered a human pathogen(111), but also attributed with crucial immune-regulatory functions. In particular, polysaccharide A from the outer membrane of *B. fragilis* was shown to direct maturation of the developing immune system by inducing the development of CD4+ (cluster of differentiation) T helper (T_H) cells and establishing a balance between T_H1 and T_H2 cell responses(180).

With that in mind, some *Bacteroides* species could also come along with favourable effects for the immune system, and the increase of certain members of *Bacteroides* could be construed as the body's attempt to counteract the detrimental effects of AD-related dysbiosis. Our observations of risen *B. dorei* in dementia and *B. uniformis* in severe dementia add further nuances to the ambivalent role of *Bacteroides* in AD.

To start with, *B. uniformis* is known to benefit the gut barrier by producing butyrate, a short chain fatty acid (SCFA) beneficial for gut barrier integrity(181,182). Moreover, research points towards its potential to ameliorate communication via the gut-brain axis as well as the metabolic and cytokine profiles in metabolic diseases such as obesity(182–187). Although some studies report the increase of *B. uniformis* in patients with anorexia nervosa(188) and mice with high-fat-diet-induced liver disease(189), the majority of results conveys a beneficial role of *B. uniformis* in health.

B. dorei, on the opposite, was also associated with AD, but not with other forms of dementia, in the study by Haran et al(111). Additionally, this bacterium is raised in autoimmune disorders and Type 1 DM (T1DM)(190).

Another *Bacteroides* species augmented in AD state according to Haran et al., but not detected in our cohort, is *B. vulgatus*(111). This pathogen is known to negatively influence neuroinflammatory signalling(191) and was previously correlated to autism(192) and T1DM as well(190).

Vogt et al. correlated the raised abundance of the genus *Bacteroides* with elevated cerebrospinal fluid (CSF) markers for AD, representing enhanced amyloid plaque and tau burden in the brains of the study participants(110).

All in all, several *Bacteroides* species enhanced in AD seem to interfere with the host's immune system and gut-brain axis, which might represent one step in the pathogenesis of AD.

4.5 Firmicutes: Loss of butyrate production and shift towards inflammation

4.5.1 Alterations in butyrate producers

Regarding the phylum Firmicutes, most taxon shifts, increases as well as decreases in AD, were observed within the families Lachnospiraceae(193) and Oscillospiraceae (formerly Ruminococcaceae)(194–196), both belonging to the order Eubacteriales within the class Clostridia. These two families include most of the known butyrate-producing bacteria(197), and indeed, the vast majority of bacteria, whose abundance was altered in our AD cohort, is involved in butyrate production.

Butyrate is a SCFA crucial for intestinal homeostasis and regulation of the immune system. It represents the major energy source for colonocytes(198), maintains the integrity of the intestinal epithelial barrier(199,200), induces satiety following meals(201), and increases mitochondrial respiration rates as well as adenosine triphosphate (ATP) production(202). Moreover, butyrate promotes sprouting of dendrites, synapse forming, and access to long-term memory(203).

Butyrate exerts its anti-inflammatory properties via different pathways: first, via suppression of the nuclear factor kappa-light-chain-enhancer of the signalling pathways of activated B cells (NF-κB)(204); second, by binding of butyrate to G protein-coupled receptors (GPRs, including free fatty acid receptor 3 (FFAR3, formerly GPR41)(205,206) and GPR109A(206,207)); third, via inhibition of histone deacetylases, which allows butyrate to regulate gene transcription(208,209).

In our study, multiple Lachnospiraceae and Oscillospiraceae taxa capable of butyrate production decreased in abundance in the gut of AD patients. These included the species *[Eubacterium] rectale*(210), two poorly described *Lachnospiraceae* species, the genera *Lachnospiraceae bacterium NK4A136 group*(211), *Agathobacter*(212), *Coprococcus 3*(213), and *Lachnospira*(214), all belonging to the family Lachnospiraceae, as well as the genus *Oscillospiraceae bacterium UCG-014*(215) of the family Oscillospiraceae. Yet also butyrate-forming bacteria aside from those two main butyrate-producing families were decreased in our AD cohort, including the genus *Erysipelotrichaceae bacterium UCG-003*(216) and the already mentioned *B. uniformis*(181,182).

Our findings align with the reduction of butyrate-synthesising bacteria in AD patients observed by Haran et al. Their findings comprised the loss of butyrate-forming *Butyrivibrio hungatei* and *proteoclasticus*, *[Eubacterium] rectale*, *Anaerobutyricum hallii* (formerly Eubacterium *hallii*), *Lachnospira eligens* (formerly *Eubacterium eligens*(217)), *F. prausnitzii*, *Clostridium sp. strain SY8519*, and *Roseburia hominis*. Moreover, they also reported that AD patients had diminished genes encoding for enzymes involved in butyrate production than elders without dementia(111).

Although not directly mentioning their capacity to produce butyrate, other studies also reported the decrease of taxa known for butyrate formation. Vogt et al. reported decreased numbers within the Oscillospiraceae family as well as of one genus belonging to the family Erysipelotrichaceae(110), which is also known to produce butyrate(218). Zhuang et al. harmonized with a diminished abundance of Lachnospiraceae, yet they contrasted the former study by reporting raised proportions of Oscillospiraceae(177).

Strikingly, our results match the latter observation since the overwhelming majority of taxa within the Oscillospiraceae family was increased in mild to severe dementia rather than in health. Moreover, also certain members of the Lachnospiraceae family behaved this way. One of the gut's main butyrate producers, the species *Faecalibacterium prausnitzii*(197), as well as its corresponding genus, and another potential butyrate-producing genus named *Lachnospira eligens group*(214) were most abundant in mild dementia. In moderate dementia, the genus *Ruminiclostridium 5*, also involved in butyrate production(219), was at its highest levels. A multitude of butyrate-producing bacteria even reached its peak abundance in severe dementia state, including the species *Anaerostipes hadrus*(220,221), *[Clostridium] leptum*(222), *Enterocloster clostridioformis*(223), *Oscillospiraceae bacterium UCG-005*(197), a species within the *Eubacterium coprostanoligenes group*(215), another group of *Faecalibacteria* named *UBA1819*(197), the genera *Eisenbergiella*(224), *Anaerotruncus*(225), *Oscillibacter*(218), and *[Eubacterium] nodatum group*(215).

On one hand, this implies that the two most abundant butyrate producers in the human gut, *E. rectale* and *F. prausnitzii*(226), are depleted in, at least, severe forms of AD. On the other hand, minor butyrate-synthesising bacteria may behave disparately and rise in number, attempting to provide functional stability. Notably, butyrate producers exhibit great biochemical diversity with different pathways and terminal enzymes. Dominating over all other pathways, the acetyl coenzyme A pathway (Ac pathway) is fuelled by carbohydrates, whereas alternative pathways are fed by proteins and represented only in minor proportions of the butyrate-producing consortium. The Ac pathway is expressed in the primary butyrate-producing species, including *E. rectale*, *F. prausnitzii*, and *Coprococcus*, while *Oscillibacter*, for instance, can produce butyrate via an alternative pathway. However, due to only minor niche overlaps, a reduction of bacteria using the Ac pathway for butyrate production can barely be outbalanced by increasing abundance of taxa expressing alternative pathways. Therefore, increases in protein-fed pathways in disease state are of minor importance and should not distract from reductions in the main pathway(218).

So far, no studies have been published that would measure the butyrate concentrations in serum or faecal samples of AD patients. In individuals with mild cognitive impairment, though, the levels of faecal butyrate were slightly but insignificantly lower than in controls(158).

In theory, a reduction in butyrate production may disturb colonocyte energy homeostasis and therefore the integrity of the intestinal epithelial barrier, facilitating bacterial translocation to the blood stream and further to the brain. There, the presence of bacteria may initiate Aβ plaque formation and neuroinflammation, causing development of AD.

To provide proof of this concept, we quantified markers for intestinal permeability, systemic inflammation, and bacterial translocation. Patients with dementia had increased DAO and sCD14 levels, indicative of intestinal barrier leakage and bacterial translocation, respectively. However, all other measurements of markers for gut barrier dysfunction, inflammation, and bacterial translocation remained inconclusive.

Nevertheless, diminished butyrate levels may be associated with the development of AD in also other ways than only via bacterial translocation.

First, post-mortem examination of brains of patients with AD revealed increased histone deacetylase levels(227), which may be due to lower butyrate production of intestinal bacteria, as butyrate is capable of inhibiting histone deacetylases(209).

Second, bacterial production of SCFAs including butyrate vitally regulates microglia maturation. GF mice displayed global defects in microglia function, which led to impaired innate immune responses and could be restored by recolonization or the mere supplementation of SCFAs(228). Also, butyrate attenuates the pro-inflammatory response of microglia to LPS via inhibition of NF-κB signalling(229). Therefore, butyrate acts as a modulator of the microglia's inflammatory response to bacteria and is crucial for a balanced neuronal milieu. Thus, the impairment of microglial Aβ clearance observed in AD patients(230) may be explained by reduced bacterial production of butyrate.

Third, several SCFAs including butyrate were shown to inhibit Aβ aggregation *in vitro*(231). *In vivo*, supplementation of sodium butyrate in an AD mouse model reduced brain Aβ levels by 40% and improved memory performance, indicated by a 25% improvement in fear response testing compared to control(232).

Fourth, diminished abundances of butyrate-producing bacteria in AD patients were associated with a decline in the anti-inflammatory P-glycoprotein pathway. The P-glycoprotein/endocannabinoid axis is the counterpart to the pro-inflammatory multidrug-resistant protein 2/hepoxilin A_3 pathway, both contributing to an equilibrium between suppression of immune response against commensals and its induction at encounter of pathogens(111). Further, the discrimination between pathogens and symbionts is mediated through regulatory T (T_{reg}) cells, whose differentiation was also shown to be facilitated by the presence of butyrate(233) as well as non-butyrate-producing immune-regulating microbiota(234). Dysbiosis may disrupt this immune homeostasis, establishing a disproportionately enhanced pro-inflammatory intestinal milieu, which may promote the development of AD(111).

4.5.2 Loss of anti-inflammatory Firmicutes

In our as well as other AD studies, various bacteria involved in the regulation of the immune system were altered in abundance. Notably, most decreases along with AD state were observed in bacteria attributed with anti-inflammatory properties, whereas the increased taxa are often referred to as pathogens or pro-inflammatory in literature.

The taxa that were significantly lost in severe AD states and associated with anti-inflammatory features are *E. rectale*(179,235,236), *Lachnospira eligens group*(236–238), *Fusicatenibacter*(239), *F. prausnitzii*(236,240–242), and *Lachnospiraceae bacterium NK4A136 group*(215,237,243–247).

E. rectale correlated positively with anti-inflammatory interleukin (IL) 10 but negatively with increased levels of pro-inflammatory cytokines in patients with mild cognitive impairment. Interestingly, increased brain amyloidosis in these patients was associated with diminished levels of IL-10 and reduced abundance of *E. rectale*(179).

L. eligens also promoted the production of IL-10 in *in-vitro* cell-based assays(238), correlated negatively with inflammatory markers such as IL-2 and CRP(236), and was reduced in patients with gestational DM(237).

In studies on IBD, *E. rectale*(235), *Fusicatenibacter*(239), and *F. prausnitzii*(240) were linked with lower degree of inflammation through IL-10 induction.

Moreover, *F. prausnitzii* led to lower production levels of IL-12, IL-8, IFN-γ, and inhibition of NK-κB signalling(240). In patients with DM, a *F. prausnitzii*-derived anti-inflammatory molecule exhibited the capacity to restore the intestinal barrier structure and function through regulating the expression of tight junction proteins(241). Fittingly, the abundance of *F. prausnitzii* was decreased in subjects with prediabetes(242).

E. rectale, *L. eligens*, and *F. prausnitzii* were all diminished in the AD cohort of Haran et al.(111) and correlated positively with lower frailty, improved cognitive function, and reduced inflammatory markers including CRP and IL-17(236). Also, *E. rectale* was inversely associated with brain amyloidosis in cognitively impaired elderly(179). Moreover, *Roseburia*, which is the genus corresponding to E. *rectale*(248), and *Faecalibacterium* both correlated negatively with CSF markers for amyloid plaque burden and tau hyperphosphorylation(158). Regarding other neurodegenerative diseases, *F. prausnitzii* was decreased in two separate cohorts of patients with Parkinson's disease(249,250).

Reduced abundance of *Lachnospiraceae bacterium NK4A136 group* in faecal microbiome was not only observed in our human AD cohort but also in a mouse model of AD(251). Generally, this species seems to interfere strongly with gut barrier integrity and glucose metabolism. In diet-induced obese mice supplemented with spermidine, the increase of *Lachnospiraceae bacterium NK4A136* due to this supplementation correlated with ameliorated intestinal integrity and reduced plasma LPS levels(211). Two other rodent studies also point towards the potential of *Lachnospiraceae bacterium NK4A136* to maintain gut barrier integrity and reduce bacterial translocation(243,244). Moreover, this species was associated with improved glucose tolerance, decreased glucose and insulin levels, and reduced inflammatory status in patients with metabolic syndrome(245) and Type 2 DM (T2DM) model mice(246). In an animal model of periodontitis, the abundance of *Lachnospiraceae bacterium NK4A136* was decreased and negatively associated with HbA_{1C}

levels(215). Also, reduced proportions of this species were reported in IBD mice, restorable by supplementation of sodium butyrate(247).
However, in a study on T1DM-induced cognitive decline, *Lachnospiraceae bacterium NK4A136* levels were raised in advanced stage type 1 diabetic mice and inversely associated with hippocampal choline(252), and its higher abundance was also observed in subjects with gestational DM(237).

Although there is no direct evidence of anti-inflammatory properties of *Agathobacter sp.*, this species was reduced not only in our AD cohort but also in subjects with prediabetes, indicative of some regulatory role within the immune system(242).

4.5.3 Gain of pro-inflammatory Firmicutes

Despite of certain AD-related Firmicutes having anti-inflammatory properties, others are associated with a pro-inflammatory status: the *Lachnospiraceae* taxa *[Ruminococcus] torques*(253,254)*,* *Enterocloster clostridioformis*(255), *Eisenbergiella*(237)*,* *Lachnoclostridium*(256,257), and *Anaerostipes hadrus*(258), the *Oscillospiraceae* taxa *Ruthenibacterium lactatiformans*(259), *Anaerotruncus*(260), and *Oscillibacter*(261)*,* as well as two other species from the order Eubacteriales named *Hungatella*(262,263) and *[Eubacterium] nodatum*(264,265).

[Ruminococcus] torques breaks down the mucus layer of the intestinal wall, converting it into SCFAs. Despite its SCFA-producing capability(253), this species is associated with a series of inflammation-related disorders and increased gut barrier permeability. In patients with IBD, disproportionate increases of *R. torques* were associated with a disrupted gut barrier, possibly through suppression of the protective mucin degrader *Akkermansia muciniphila* by *R. torques*(254).
The pathogen *Enterocloster clostridioformis* (formerly *Clostridium clostridioforme*) can cause severe infections including bacteremia, sepsis, and meningitis(255). Its structurally narrow relative *Eisenbergiella*(224), which was also significantly increased in severe AD stage, raised during first trimester of patients with gestational DM(237).
The genus *Lachnoclostridium* was increased in obese women with polycystic ovary syndrome(256) and in patients with diabetic peripheral neuropathy(257). While decreased

proportions of *Lachnoclostridium* were found in AD patients(111,177), its abundance was raised in other dementia types(111).

Anaerostipes hadrus is suggested to play a major role in butyrate production, thereby benefitting the host(221). Moreover, *A. hadrus*(111), *A. caccae*(177), and the genus *Anaerostipes*(266) were associated with health rather than with AD in human cohort studies. Also, *A. hadrus* correlated positively with improved cognitive function, lower frailty, and reduced inflammatory markers(236). However, in a mouse model of colitis, administration of the subtype *A. hadrus* even accelerated dysbiosis and aggravated colitis, whereas it did not shift bacterial composition or cause colitis in healthy mice. Thus, the potentially beneficial *A. hadrus* may exert detrimental effects to health when surrounded by a dysbiotic environment(258).

The species *Ruthenibacterium lactatiformans* was increased in patients with progressive multiple sclerosis, a neuroinflammatory autoimmune disease that may be triggered by both a lack of beneficial microbes that regulate autoimmunity and an overabundance of pro-inflammatory bacteria(259). However, its abundance was diminished in patients with rheumatoid arthritis, another autoimmune inflammatory disease(267).

Anaerotruncus is commonly seen as a pathogen(260) and was increased in two other human AD cohorts(177,266) and one PD cohort(268). Further, its faecal abundance augmented upon Aβ-injection into the brains of model mice, as did the numbers of the genus *Osillibacter*(261).

The number of *Hungatella* (formerly known as Clostridium hathewayi(269)) correlated positively with the amount of T helper 17 cells in the intestine(262), a pro-inflammatory subspecies of T helper cells playing the key role in the immune system's defence against extracellular pathogens(270). Moreover, *Hungatella* mediates promoter hypermethylation of tumour suppressor genes in host colonic epithelial cells during development of colorectal cancer(263).

[Eubacterium] nodatum is a periodontal pathogen implicated in periodontitis. While high abundance of *E. nodatum* in oral microbiome is associated with generalized aggressive periodontitis among young individuals(264) and with chronic periodontitis in adults in general(265), high serum anti-*E. nodatum* immunoglobulin G (IgG) titres were related to lower risk for periodontitis(271). The observation of an increased periodontal pathogen in our severe AD group matches the reports of periodontitis to convey augmented risk of developing AD(272). Notwithstanding, high anti-*E. nodatum* IgG was correlated to lower

risk of AD in Noble et al.(273). Taken together, these results underscore the complex role of host immune response to pathogens in development of AD.

4.5.4 Countermotion: Firmicutes counteracting inflammation in AD

Similarly to the rise of certain butyrate producers among the shades of deteriorated overall butyrate production, a small number of anti-inflammatory gut bacteria seem to act inversely to the built-up scheme: They increase in number in order to counteract the pro-inflammatory milieu associated with AD.

Four anti-inflammatory taxa were increased rather than decreased in moderate to severe AD. While all of them belong to the phylum Firmicutes, only one of them, *[Clostridium] leptum*, ranks amongst the order of Eubacteriales, which comprises most of the immune-modulatory species mentioned in the chapters above. The other three are members of the order Lactobacillales and class Bacilli: *Lactobacillus amylovorus*, *Streptococcus salivarius*, and *S. thermophilus*.

To start with, *C. leptum* promotes immune maturation and increases T_{reg} cell numbers to alleviate the development of asthma(274). Also, it is also capable of butyrate production(222) and its reduced abundance was reported in IBD(275). Raises of *C. leptum* were linked to severe AD.

Increases of *Lactobacillus amylovorus* and its corresponding genus *Lactobacillus*, family Lactobacillaceae, order Lactobacillales, and class Bacilli were associated with moderate AD. These findings are of particular interest since the species *Lactobacillus* is generally assigned with benefits for neurodegenerative diseases including AD. Administration of probiotic *Lactobacillus* strains increased neurotransmitter levels in regions of the brain typically affected by AD and decreased cognitive dysfunction, inflammation in the gut, and levels of circulating inflammatory cytokines in mice(276). *L. amylovorus* in particular was recognized as the most potent *Lactobacillus* in removal of ammonia from the intestine, thereby also limiting blood ammonia levels(277). Previous research had shown that blood ammonia levels in AD were significantly increased compared to control, implying that the neurotoxic nature of ammonia may contribute to AD development(278). Moreover, *L. amylovorus* could counteract inflammation triggered by enterotoxigenic *Escherichia coli* (ETEC) through inhibition of the toll-like receptor 4 (TLR4) signalling pathway and through cytokine regulation. Since pathogens can initiate intestinal inflammation via binding of LPS or heat shock proteins to TLR4 and consecutive cytokine over-production, inhibition of this pro-inflammatory pathway represents a powerful trait of probiotic species to counteract pathogen-induced damage(279).

Also belonging to the order Lactobacillales, raises of *Streptococcus salivarius* and *S. thermophilus* were correlated to severe dementia state. *Streptococcus* species are commensals of the oral microbiome but can also inhabit the small intestine(280). Both, *S. salivarius* and *S. thermophilus*, are commonly used as probiotics and attributed with anti-inflammatory properties: *S. salivarius* inhibits the activation of pro-inflammatory pathways including NF-κB(281) and PPARγ (peroxisome proliferation activated receptor)(282) in intestinal epithelial cells and significantly prevents inflammation in colitis mouse models(281). Colitis was also suppressed by *S. thermophilus* through amelioration of gut barrier function and reduction of bacterial translocation into the colonic tissue(283,284). Therefore, the association of both *Streptococci* to severe AD in our study remains unclear.

Finally, one of our findings also suggests the decrease of a certain pro-inflammatory genus in AD, namely *Erysipelotrichaceae bacterium UCG-003*. Despite its potential ability for producing butyrate(216), increased abundance of Erysipelotrichaceae has been associated with colorectal cancer(285) and tumour necrosis factor (TNF)-driven Crohn's disease-like inflammation(286). Moreover, some Erysipelotrichaceae bacteria were able to induce pro-inflammatory T_H17 cells in the intestinal wall(262). Although the *UCG-003* subtype was associated with healthy ageing(287), distinct functional roles for this subtype have not been reported yet, leaving its role in AD development enigmatic.

4.6 Proteobacteria: Raise of pro-inflammatory pathogens

The pro-inflammatory burden observed in AD may not only be driven by pro-inflammatory Firmicutes species but also by taxa belonging to other phyla.

In our as well as in other AD studies, taxa within the phylum Proteobacteria were enriched. In particular, the family Enterobacteriaceae and corresponding order Enterobacteriales raised in abundance in our severe AD group, which is reflected by findings in another human

AD cohort(266) as well as in patients with mild cognitive impairment(158). An increase in *Bilophila* genus also belonging to Proteobacteria was observed by Vogt et al. in AD(110). In both of the latter studies, those bacteria were associated with increased CSF markers of AD pathology, including markers for amyloid plaque burden and tau hyperphosphorylation(110,158). Moreover, the increase in Proteobacteria was associated with a higher number of the AD risk allele APOE ε4(266).

Many human pathogens belong to the phylum Proteobacteria, such as Enterobacteriaceae, Pseudomonaceae and Vibrionaceae families. The cell wall of those gram-negative bacteria is enriched for LPS, which induces the production of pro-inflammatory cytokines(288) and further exacerbates AD pathology in brains of mice when translocated to the blood stream(289). Notably, the endotoxic activity of LPS from bacteria belonging to Proteobacteria is considered far higher than that from *B. fragilis* mentioned above(290).

Klebsiella pneumonia, a human pathogen belonging to the family Enterobacteriaceae, raised in abundance in AD patients compared to control(111). Moreover, the abundance of *Escherichia/Shigella*, other members of Enterobacteriaceae, correlated positively with brain amyloidosis in patients with mild cognitive impairment(179). *Escherichia* possess the ability to induce production of pro-inflammatory mediators through a NLRP3 (NLR family pyrin domain containing 3) dependent mechanism(291). Given that the NLRP3 inflammasome can promote the formation of protein aggregates including Aβ(292), and that NLRP3 inflammasome deficiency in an AD mouse model resulted in diminished release of pro-inflammatory cytokines and reduced Aβ deposition(293), it would be unsurprisingly if *Escherichia* contributed to the formation of Aβ plaques in AD pathogenesis via an NLRP3 inflammasome-dependent mechanism.

4.7 Actinobacteria: Raises of pathobionts and drops of Bifidobacteria

Taxa proportions in yet another phylum were altered in our AD cohort. Pathobiontic members of the phylum Actinobacteria raised in abundance in the gut microbiome of AD patients, namely the species *Collinsella aerofaciens* and the family Actinomycetaceae as well as its corresponding order Actinomycetales.

C. aerofaciens was not only increased in our AD cohort but also in that of Haran et al.(111), and another species named *C. intestinalis* was associated with AD by Zhuang et al.(177). Although not much is known about the distinct features of *C. aerofaciens*, its genus, *Collinsella*, is described as a lactate-producing pathobiont(294) and has been associated with T2DM(295), rheumatoid arthritis(296), and symptomatic atherosclerosis(297). Further, administration of *Collinsella* impairs gut barrier integrity by reducing the expression of tight junction proteins and increases pro-inflammatory IL-17 network cytokines(296).

The Actinomycetaceae family comprises several human pathobionts, of which the genus *Actinomyces* is clinically the most important one. *Actinomyces* inhabits the gums and causes infections in dental procedures as well as actinomycosis, characterized by the formation of abscesses in the mouth and other body regions(298). High serum anti-*A. naeslundii* titre was associated with doubled risk of AD(273).

In total, our results concerning the Actinobacteria phylum meet the observation of Zhuang et al., who reported a slight increase in these phylum and class in AD patients(177). However, Vogt et al. described a drop in Actinobacteria, which they attributed to a decreased abundance of the genus *Bifidobacterium*(110).

When comparing other forms of dementia with AD, diminished numbers of *B. bifidum*, yet an increased *B. longum* count could discriminate AD from others(111). In our cohort, *Bifidobacteria* did not differ between dementia and control patients; however, as Bifidobacteriales and Actinomycetales belong to the same class, the Actinomycetia, a relative decrease in *Bifidobacteria* in our cohort might be concealed by the relative increase of Actinomycetaceae. Alternatively, the use of the hypervariable regions V1-V2 in 16S sequencing may reduce the recovery rate of Bifidobacteria, thereby obscuring changes in this taxon between groups.

Members of *Bifidobacterium* genus possess anti-inflammatory properties, including IL-10 induction, and improve gut barrier function by increasing the expression of tight junction proteins(299). Moreover, *Bifidobacterium* has been reported to decrease LPS levels in the murine intestine(300) as well as bacterial translocation to the blood stream(178). Notably, the administration of *Bifidobacteria* strains in various AD rodent models repeatedly resulted in the reduction of Aβ deposition and neuroinflammation in cortex and hippocampus as well as the amelioration of memory deficits(163,301–304). Further, *Bifidobacteria* are considered one of the main γ-amino butyric acid (GABA) producers among bacteria(305) and may therefore contribute to the dysfunction in GABAergic signal transmission observed in AD(306,307). Strengthening this thesis, GABA levels were significantly decreased in faeces of mice transplanted with stool from an AD patient compared to control group mice(161).

In addition, Bifidobacteria take part as so-called first degraders in the multistep process of butyrate production. The degradation of resistant starch to butyrate relies on the cross-feeding of fermentation products between two groups of bacteria. While first degraders like *Bifidobacteria* take down resistant polysaccharides into smaller products like acetate, lactate, and mono- or oligosaccharides, second degraders can thereafter degrade those to butyrate(197). Since butyrate producers rely on the presence of first degraders, the decline of *Bifidobacteria* observed by Vogt et al. fits perfectly into the schema of reduced butyrate production promoting AD pathogenesis.

4.8 Influence of malnutrition on microbiome composition in AD

Malnutrition is commonly observed in dementia(111,308–311) and aggravates the loss of cognitive capacities(308). Not only does weight loss precede symptomatic dementia(310), but it also worsens with severity and progression of the disease(311).

Even though all patients in our AD cohort were treated as per nutritional support standards, more than three quarters were malnourished according to MNA-SF scores. Therefore, it is impossible to distinguish between malnutrition and dementia as the starting point of the observed dysbiosis. However, our multivariate analysis suggests that malnutrition is the main driver of microbiome alterations in dementia since BMI emerged as one of the strongest influencing factors.

It does not come as a surprise that malnutrition in the elderly influences microbiome composition(312). Remarkably, many of the taxon shifts observed in our AD cohort mirror changes explicitly connected to low dietary intake of carbohydrates, especially of resistant starch. For instance, *Eisenbergiella*, which was increased in faecal microbiome of our AD patients, commonly emerges in hosts subjected to a diet low in carbohydrates(313). Another bacterium enriched in our AD cohort is *Collinsella*, which is incapable of fermenting dietary fibres, thus only growing during a diet of low fibre intake(314).

Simultaneously, dietary fibre is needed for microbial production of butyrate. As a result, various butyrate producers were underrepresented in the faecal microbiome of patients suffering from anorexia nervosa(315). In AD patients as well, the reduction in butyrate-producing bacteria may simply reflect reduced carbohydrate and resistant starch intake. Following this theory, reduced fibre intake may harm gut barrier integrity and elicit dysbiosis, thereby laying the foundation for the development of AD.

However, undernutrition in AD patients oftentimes occurs even with accurate intake of macronutrients(316). Thus, it remains unclear to what extent dietary habits interfere with development of AD.

4.9 Polypharmacy in AD and consequences on microbiome composition

Besides malnutrition, prescription drug use was the second main predictor of gut dysbiosis in our AD patients. Patients in the AD cohort were prescribed on average three times more drugs than control participants. Growing evidence points towards the extensive effects of drug use on gut microbiome composition. Strikingly, not only antibiotics but members of all therapeutic classes elicit microbiome changes in the human gut(317). Our pilot study presents additional data on drug influence on the gut microbiome, which yet must be treated with caution due to low sample size.

As expected, the impact of specific drug classes on the general community structure, including alpha and beta diversity, was minor. However, each class was associated with certain changes on low taxonomic levels. Especially the use of PPIs shaped the bacterial composition in our cohort, yielding the growth of oral bacteria in the intestine. This finding overlaps with previous reports of increased oral bacteria due to PPI use(318). Also, analysis of drug impact on biomarkers for intestinal inflammation, gut permeability, and microbial translocation revealed enhanced gut inflammation in association with PPI use. Additionally, the use of antihypertensive was correlated to slightly yet significantly increased CRP levels. Nonetheless, the latter finding likely reflects the underlying disease rather than the use of antihypertensives itself, since arterial hypertension is linked to raised CRP levels(319).

All in all, these minor additions on drug interference with microbiome composition strengthen the validity of our findings despite low sample size.

4.10 Challenges in comparison between human microbiome AD studies

When comparing our study to others on human microbiome involvement in AD, several structural differences must be considered.

First of all, our study cohort comprises rather severely diseased individuals, whereas the majority of the compared studies were carried out in patients with either mild cognitive impairment(158,179) or mild to moderate dementia(110,177,320). A shift in microbiome composition from early to advanced disease state may therefore account for the stated discrepancies between our results and those of others. To our knowledge, only one other research group observed patients with mostly severe dementia, whose results fit very well to ours(111).

Additionally, we shed first insights in sequencing-based AD-associated microbiome changes in European patients, whereas others focussed on US American(110,111) or Chinese(177) cohorts. Of those two studies carried out in Europe, however, one observed solely patients with mild cognitive impairment(179) and the other did not carry out any bacterial sequencing but analysed exclusively certain target species(320).

In our study, the sequencing depth of every sample was sufficiently high, so that none of the samples had to be excluded. Nevertheless, the 16S rRNA-based sequencing technique used in our study trails the method of shotgun sequencing used by Haran et al.(111), which reveals more sequence-based information about microbial diversity and sheds additional light on bacterial functionality via metagenomic sequencing(321). The approach applied in our study by PICRUSt to predict bacterial functions based on knowledge about related species represents only an approximation to the actual metagenome and results must be treated with caution.

4.11 Limitations

This pilot study has multiple limitations, above all those generally met by microbiome studies. For ethical and practical reasons, faecal matter is usually used as substrate for microbiome analysis(321). However, the stool microbiome composition only reflects bacteria occupying the gut lumen but not those adherent to the mucus covering the intestinal epithelium. Thus, information about mucus-resident microbiota, which may be differentially altered in disease state, fails to be conveyed in microbiome analyses of stool samples(322). Moreover, the composition of microbiota varies considerably between individuals and to some degree also in each individual over time(166). This poses the challenge to divide inter- and intraindividual variations from disease defining differences in microbiome structure.
Yet, the high diversity of the microbiome comes along with the presence of a large fraction of unexplored microorganisms. To date, around 10% of metagenomic reads of any given gut microbiome sample are still not mappable to specific species(323–325). Identification of such organisms as differentially abundant in disease state does not bring along any additional value at present, as their features and potential impact to disease development remain elusive. Further, although bacteria represent the overwhelming majority of the microbial genome, with over 99% of genes originating from bacteria(326,327), the gut habitats also a multitude of other microorganisms like viruses, archaea, and microbial eukaryotes, including fungi, yeasts, protozoa, flagellates, and ciliates(328,329). Despite comprising only a small fraction of the microbial genome, they play crucial roles within the ecosystem and in the onset of diseases(330), as demonstrated by the involvement of viruses in the formation of amyloid plaques. Thus, limiting the observation span to bacteria addresses only one participant in the interplay of microorganisms and can never reveal the impact of the whole microbiome in disease development.

Focussing on this microbiome study, one limitation consists in the choice of allocation to either dementia or control cohort. Although diagnosis of dementia performed by a board-certified neurologist or psychiatrist was stated in the inclusion parameters, MMSE values as low as those in mild dementia state were observed in some controls. In order to adequately represent changes in the course of cognitive decline, MMSE scores lower than 27 were valued as a sign for beginning cognitive impairment and were therefore attributed to the dementia group. Still, MMSE scores are not sufficient to diagnose any form of dementia since diagnosis requires a thorough neurological examination and should meet the ICD-10 criteria.

Several difficulties evolved from our study participants' lack of cognitive ability. First, the gold standard for gut barrier permeability testing, the differential sugar-absorption test(331), could not be carried out, as the patients' low cognitive ability would result in the disproportional need for a urinary catheter. Instead, intestinal epithelial permeability was evaluated by measuring surrogate parameters in blood and faeces. Secondly, blood tests apart from routine laboratory could not be carried out in patients missing informed consent and stool sampling was impossible in others due to the underlying forgetfulness, which decreased sample size for certain data points.

This leads to the foremost limitations of our study: the pilot study design and the concomitant low total number of study participants, further diminished out of reasons stated above. To increase explanatory power of the results, like the tendency towards reducing alpha diversity with decreasing cognitive function, a higher sample size would have been needed. However, our data still clearly points out the presence of changes in microbiome composition accompanying AD development and its alignment with other AD microbiome studies further strengthens our results.

Due to the observational study design, multiple methodical changes in data analysis have been accepted in order to get the most value out of the data set. These included a switch between two different interfaces for microbiome analysis, Galaxy and Calypso 7.14, since either certain microbiome parameters could not be computed in one of the interfaces or one interface had a more feasible, hence, favourable calculation algorithm. Nonetheless, repeated changes of analysis strategies and tools increase observational bias. Further, subgroup analyses lack significance due to even lower sample size, yet they yield results resembling those already published, which again fortifies our data set.

Finally, our study provides only cross-sectional data, which cannot shed any light on questions regarding a possible cause-effect relationship between malnutrition, microbiota, and neurodegeneration.

4.12 Possible cause-effect relationship between microbiota and AD

Within the last years, efforts have been made to answer this “chicken-and-egg” dilemma. Decreased Aβ plaque burden compared to control was shown in GF AD mice(109), in colonised AD mice treated with antibiotics(332) as well as with anti-inflammatory probiotic species(163), whereas transplantation of faeces from an AD mouse model to wild type mice induced Aβ plaque and NFT formation in the brain of those animals(165). Moreover, GF mice transplanted with faecal samples from an AD patient exhibited memory deficits and reduced faecal metabolites related to the nervous system compared to mice that received faeces from a healthy volunteer(161). These mouse studies strongly point towards the origin of neurodegeneration in the gut. Yet, the gut-brain-axis always works in a bidirectional manner, which virtually guarantees downstream effects of any impaired neuronal system on intestinal microbiota. Thus, more experimental and longitudinal observational studies are required to be able to assign causality to gut microbiome changes in the development of AD.

4.13 Future directions

Supposed that changes in microbiome composition could not only elicit but also delay cognitive dysfunction in AD elderly, probiotic intervention might emerge as a potential therapeutic strategy in AD treatment. Especially the use of butyrate-producing bacterial strains will be of particular interest. First studies focusing on this purpose already achieved promising results with increases in cognitive ability and decreased markers of systemic inflammation(333). Furthermore, the influence of dietary changes on AD development remains to be assessed.

4.14 Conclusion

In summary, our study aligns with a multitude of studies in animal AD models as well as patients with AD suggesting distinct microbial profiles implicated in AD pathogenesis. Evidence points towards a rise in pro-inflammatory and drop in anti-inflammatory taxa, contributing to low-grade systemic and subsequently neuronal inflammation that impacts on neuronal function. Further, the loss of butyrate-producing species may result in a leaky-gut like intestinal barrier impairment, enabling microbiota and microbial particles to enter the blood stream and travel up to the brain. Once the inflammatorily destructed blood brain barrier is crossed, the microbiota may damage brain neurons either directly or through inducing additional pro-inflammatory immune responses. Eventually, the microorganisms may be trapped in amyloid plaques, which would set of a cascade of tau formation and further neurodegeneration. This all implies that AD is the neuropathological expression of the immune system's persistent loss of balance, with the microbiome either initiating or, at least, potentiating inflammatory burden via the gut-brain-axis.

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Appendix

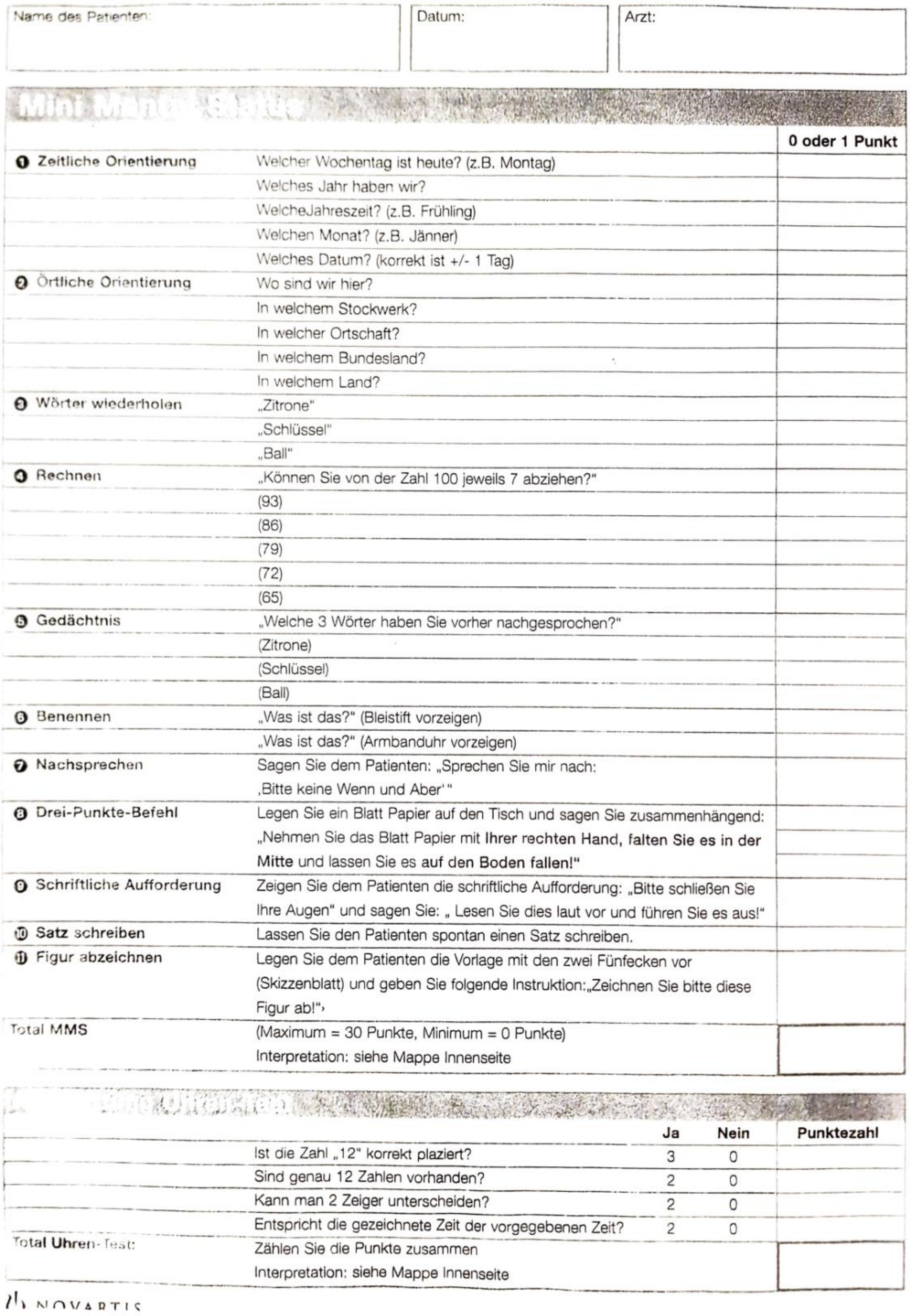
Appendix A – MMSE and clock-drawing test adapted by Novartis………….……..A-1

Appendix B – Already released publications………………………………………B-1

B1 Abstract presentation at the 53rd annual meeting of the Austrian Society for Gastroenterology and Hepatology (Österreichische Gesellschaft für Gastroenterologie und Hepatologie, ÖGGH). ……………………………………………………………………......B-1

B2 Research article in BMC Geriatric……………………....…………………….B-3

Appendix A – MMSE and clock-drawing test adapted by Novartis



Name des Patienten:	Datum:	Arzt:

Mini Mental Status

		0 oder 1 Punkt
❶ **Zeitliche Orientierung**	Welcher Wochentag ist heute? (z.B. Montag)	
	Welches Jahr haben wir?	
	WelcheJahreszeit? (z.B. Frühling)	
	Welchen Monat? (z.B. Jänner)	
	Welches Datum? (korrekt ist +/- 1 Tag)	
❷ **Örtliche Orientierung**	Wo sind wir hier?	
	In welchem Stockwerk?	
	In welcher Ortschaft?	
	In welchem Bundesland?	
	In welchem Land?	
❸ **Wörter wiederholen**	„Zitrone"	
	„Schlüssel"	
	„Ball"	
❹ **Rechnen**	„Können Sie von der Zahl 100 jeweils 7 abziehen?"	
	(93)	
	(86)	
	(79)	
	(72)	
	(65)	
❺ **Gedächtnis**	„Welche 3 Wörter haben Sie vorher nachgesprochen?"	
	(Zitrone)	
	(Schlüssel)	
	(Ball)	
❻ **Benennen**	„Was ist das?" (Bleistift vorzeigen)	
	„Was ist das?" (Armbanduhr vorzeigen)	
❼ **Nachsprechen**	Sagen Sie dem Patienten: „Sprechen Sie mir nach: ‚Bitte keine Wenn und Aber' "	
❽ **Drei-Punkte-Befehl**	Legen Sie ein Blatt Papier auf den Tisch und sagen Sie zusammenhängend: „Nehmen Sie das Blatt Papier mit **Ihrer rechten Hand, falten Sie es in der Mitte** und lassen Sie es **auf den Boden fallen!"**	
❾ **Schriftliche Aufforderung**	Zeigen Sie dem Patienten die schriftliche Aufforderung: „Bitte schließen Sie Ihre Augen" und sagen Sie: „ Lesen Sie dies laut vor und führen Sie es aus!"	
❿ **Satz schreiben**	Lassen Sie den Patienten spontan einen Satz schreiben.	
⓫ **Figur abzeichnen**	Legen Sie dem Patienten die Vorlage mit den zwei Fünfecken vor (Skizzenblatt) und geben Sie folgende Instruktion:„Zeichnen Sie bitte diese Figur ab!"›	
Total **MMS**	(Maximum = 30 Punkte, Minimum = 0 Punkte) Interpretation: siehe Mappe Innenseite	

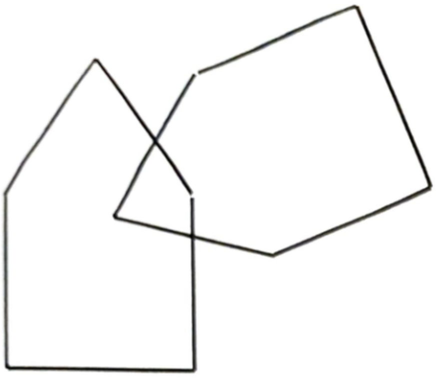
[illegible]

		Ja	**Nein**	**Punktezahl**
	Ist die Zahl „12" korrekt plaziert?	3	0	
	Sind genau 12 Zahlen vorhanden?	2	0	
	Kann man 2 Zeiger unterscheiden?	2	0	
	Entspricht die gezeichnete Zeit der vorgegebenen Zeit?	2	0	
Total **Uhren**-Test:	Zählen Sie die Punkte zusammen Interpretation: siehe Mappe Innenseite			

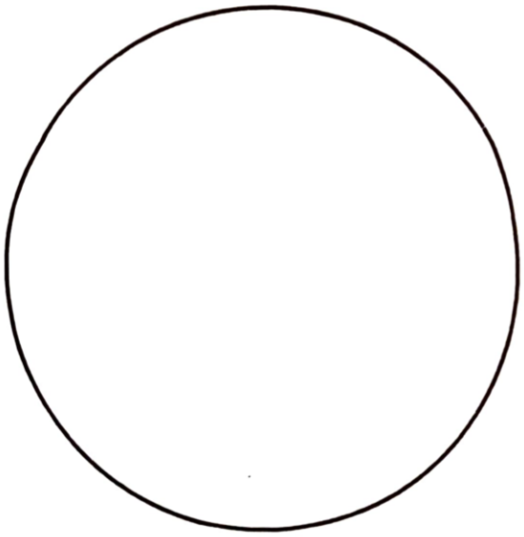
NOVARTIS

Bitte schließen Sie Ihre Augen!

PatientIn:__________________Untersucher:____________Datum:_________Station:____



11:10



Mini Mental Status – ergänzende Angaben zum Test

	Sie und Ihre Patientin/Ihr Patient benötigen einen Bleistift und zwei leere Blätter (nicht liniert oder kariert).
	Jede richtige Antwort gibt einen Punkt.
❶ Zeitliche Orientierung:	Falls keine Antwort, raten lassen.
❷ Örtliche Orientierung:	Falls keine Antwort, raten lassen.
❸ Drei Wörter wiederholen:	Sprechen Sie die drei Wörter im Rhythmus 1 pro Sekunde vor. Lassen Sie Ihren Patienten die drei Wörter wiederholen. Werden im ersten Versuch nicht alle drei Wörter richtig nachgesprochen, wiederholen Sie diese bis zu sechs Mal, bis alle Wörter gelernt sind. Wichtig: nur die erste Antwort bewerten.
❹ Selbständig rechnen:	Ihr Patient soll von der Zahl 100 jeweils 7 subtrahieren. Pro korrekter Subtraktion gibt es 1 Punkt. Wird aber von einem falschen Zwischenresultat 7 korrekt abgezogen, geben Sie wieder einen Punkt.
❺ Gedächtnis:	Die genannte Reihenfolge spielt keine Rolle.
❽ Drei-Punkte-Befehl:	Legen Sie ein Blatt Papier auf den Tisch und geben Sie den Befehl zusammenhängend. Jede korrekt ausgeführte Handlung gibt einen Punkt.
❾ Schriftliche Aufforderung:	Die Durchführung gibt einen Punkt.
❿ Satz schreiben:	Lassen Sie den Patienten selbständig einen vollständigen Satz schreiben (mind. 1 Subjekt, 1 Verb). Schreibfehler nicht beachten.
⓫ Figur abzeichnen:	Nur ein Versuch! Alle Ecken/Winkel müssen vorhanden sein, ebenso müssen sich zwei Ecken wie in der Originalzeichnung überschneiden. Zittrige Linien oder eine Rotation der Figur nicht berücksichtigen.

Uhren-Test – ergänzende Angaben zum Test

Bitten Sie Ihren Patienten das Ziffernblatt einer Uhr in den Kreis einzuzeichnen. Die Zeiger sollen die Zeit 11:10 (10 Minuten nach 11) anzeigen

Interpretation Mini Mental Status

Unter 27 Punkte:	Erreicht der Patient weniger als 27 Punkte, ist eine eingehende Untersuchung angezeigt.
27 bis 30 Punkte:	Aufgrund des Resultats ist noch keine weitere Abklärung angezeigt. Trotzdem kann aufgrund des klinischen Urteils eine eingehende Untersuchung indiziert sein.

Interpretation Uhren-Test

0 bis 6 Punkte:	Eine eingehende Untersuchung ist angezeigt.
7 bis 9 Punkte:	Aufgrund des Resultats ist noch keine weitere Abklärung angezeigt. Trotzdem kann aufgrund des klinischen Urteils eine eingehende Untersuchung indiziert sein.

Appendix B – Already released publications

B1 Abstract presentation at the 53rd annual meeting of the Austrian Society for Gastroenterology and Hepatology (Österreichische Gesellschaft für Gastroenterologie und Hepatologie, ÖGGH)

Engertsberger L, Horvath A, Komarova I, Feldbacher N, Leber B, Pichler G, et al. Dysbiosis in dementia is associated with gut barrier dysfunction and linked to malnutrition and prescription drug use. Z Gastroenterol. 2020;58(05): P29. https://doi.org/10.1055/s-0040-1712280.

Background Dementia is an increasing public health threat worldwide. The pathogenesis of dementia has not been fully elucidated yet. Inflammatory processes are hypothesized to play an important role as a driver for cognitive decline but the origin of inflammation is not clear. We hypothesize that disturbances in gut microbiome composition, gut barrier dysfunction, bacterial translocation and resulting inflammation are associated with cognitive dysfunction in dementia.

Methods To test this hypothesis, a cohort of 23 patients with dementia and 18 age and sex matched controls without cognitive impairments were studied. Gut microbiome composition, gut barrier dysfunction, bacterial translocation and inflammation were assessed from stool and serum samples. Malnutrition was assessed by Mini Nutritional Assessment Short Form (MNA-SF), additionally, detailed information on drug use was collected. Microbiome composition was assessed by 16S sequencing, QIIME 2 and Calypso 7.14 tools.

Results Dementia was associated with dysbiosis characterized by differences in beta diversity and changes in taxonomic composition. Potentially butyrate producing bacteria, including Eubacterium rectale and members of the Lachnospiraceae genus, were less abundant in demented individuals. Gut permeability was increased as evidenced by increased serum diamine oxidase levels, and systemic inflammation was confirmed by increased soluble cluster of differentiation 14 levels (sCD14). BMI and statin use influenced microbiome composition. Notably, demented individuals took three times more prescription drugs than control, and three quarters of this group were malnourished.

Conclusion Dementia is associated with changes in gut microbiome composition and increased biomarkers of gut permeability and inflammation. Demented individuals harbored less potentially butyrate producing bacteria, further promoting butyrate as a link between dysbiosis, gut barrier dysfunction and cognitive decline. Moreover, malnutrition and drug intake were factors impacting microbiome composition. Taken together, increasing butyrate producing bacteria and targeting malnutrition may present promising therapeutic targets in dementia.

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Dysbiosis, gut barrier dysfunction and inflammation in dementia: a pilot study

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Abstract

Background: Dementia is an increasing public health threat worldwide. The pathogenesis of dementia has not been fully elucidated yet. Inflammatory processes are hypothesized to play an important role as a driver for cognitive decline but the origin of inflammation is not clear. We hypothesize that disturbances in gut microbiome composition, gut barrier dysfunction, bacterial translocation and resulting inflammation are associated with cognitive dysfunction in dementia.

Methods: To test this hypothesis, a cohort of 23 patients with dementia and 18 age and sex matched controls without cognitive impairments were studied. Gut microbiome composition, gut barrier dysfunction, bacterial translocation and inflammation were assessed from stool and serum samples. Malnutrition was assessed by Mini Nutritional Assessment Short Form (MNA-SF), detailed information on drug use was collected. Microbiome composition was assessed by 16S rRNA sequencing, QIIME 2 and Calypso 7.14 tools.

Results: Dementia was associated with dysbiosis characterized by differences in beta diversity and changes in taxonomic composition. Gut permeability was increased as evidenced by increased serum diamine oxidase (DAO) levels and systemic inflammation was confirmed by increased soluble cluster of differentiation 14 levels (sCD14). BMI and statin use had the strongest impact on microbiome composition.

Conclusion: Dementia is associated with changes in gut microbiome composition and increased biomarkers of gut permeability and inflammation. *Lachnospiraceae NK4A136 group* as potential butyrate producer was reduced in dementia. Malnutrition and drug intake were factors, that impact on microbiome composition. Increasing butyrate producing bacteria and targeting malnutrition may be promising therapeutic targets in dementia.

Trial registration: NCT03167983.

Keywords: Microbiome, Diversity, Gut barrier, Inflammation, Cognitive function, Butyrate producer

Background

Dementia leads to disability and dependency among older people worldwide and thereby has enormous physical, psychological, social and economic impact on patients, caregivers, families and society [1]. Alzheimer's disease (AD) is the most common form of dementia accounting for 60–70% of the cases [1]. In AD, pathologic protein aggregates and neuroinflammation mediated by microglia cells are involved in the pathogenesis, however, the exact mechanism is still unclear [2]. Microglia maturation and function is critically dependent on short-chain fatty acids produced by the gut microbiome and therefore highlights the microbiome as a potential diagnostic and therapeutic target in dementia [3]. During ageing, the gut microbiome decreases in diversity, loses beneficial taxa and facultative pathogens increase [4].

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Diet and the place of residence play an important role in the shaping of the microbiome [5, 6]. Ageing is also associated with inflammation – often termed as "inflammageing". Inflammation is further associated with an increase in gut permeability, mucosal inflammation and bacterial translocation [2].

Since the main risk factor for developing dementia, especially AD, is ageing, it can be hypothesized that the gut-brain axis is a possible link between age and disease related dysbiosis and inflammation. Animal studies suggest that AD is associated with changes in the gut microbiome composition with a decrease in beneficial, anti-inflammatory genera [7]. Furthermore, genetic alterations in amyloid genes can influence microbiome composition in mice, pointing towards a vicious cycle in AD development [8]. Recently studies from the USA have identified a loss in species diversity and differences in bacterial composition in the stool of AD patients compared to matched controls [9]. A study from Japan has also shown that gut microbiome composition is independently and strongly associated with dementia [10]. Furthermore, it has been recently published that the microbiome of dementia patients causes a dysregulation of the anti-inflammatory P-glycoprotein pathway [11].

So far factors that may influence the composition of the gut microbiome in patients with dementia have not been studied in detail. Potentially influencing factors could be malnutrition, which is common in dementia and associated with disease severity, [12–14] or drug intake, since polypharmacy is a common problem in elderly persons and the impact of drugs on the microbiome has recently gained attention [15–17].

We hypothesize that dementia is associated with dysbiosis, gut barrier dysfunction and inflammation and we aim to identify external factors influencing microbiome composition in dementia, such as nutrition and drug intake. To study this, we conducted a prospective controlled cohort study in patients with dementia and age matched controls.

Methods

Between July 2017 and March 2018 we recruited 25 patients with diagnosis of Alzheimer type ($n = 21$) or mixed type (Alzheimer type plus vascular type, $n = 4$) dementia with a Mini Mental State Examination (MMSE) ≤ 26 and 18 age and sex matched controls without evidence of dementia and a MMSE > 26 at the Albert-Schweitzer Hospital Graz and at the University Hospital Graz. Diagnosis of dementia was made by a board-certified neurologist/psychiatrist and according to ICD10 criteria including cerebral imaging and exclusion of differential diagnosis by full laboratory workup. Participants *or* their legal representative gave written informed consent. We excluded participants with other forms of dementia, inflammatory bowel diseases, liver cirrhosis or recent (< 4 weeks) antibiotic or probiotic treatment. The study (29–420 ex 16/17) was approved by the ethics committee Ethic Committee of the Medical University of Graz (IRB00002556) and has been registered at clinicaltrials.gov (NCT03167983) before the study started. The study was performed according to the Declaration of Helsinki and Good Clinical Practise guidelines. Written informed consent was obtained before any study specific procedure was performed from participants or their legal representatives (in case patients were not able to give written consent any more due to the severity of cognitive dysfunction). Routine blood biochemistry analysis including full blood count, electrolytes, renal function, liver function, albumin and total protein levels and inflammation parameter and a detailed medical history was performed. Stool and serum samples were collected for analysis of gut microbiome composition and biomarkers of intestinal permeability, inflammation and bacterial translocation. Serum samples were collected after overnight fasting. Stool samples were collected by the patients or caregivers in sterile collection tubes either on the same day or the evening before the study visit. Samples were kept on 4 °C until arrival at the hospital and then frozen immediately at − 80 °C. Mini Nutritional Assessment Short Form [18] was used to assess nutritional status.

Cognitive function

The Mini-Mental State Examination [19] and the clock drawing test [20] were used to quantify cognitive function. We classified cognitive dysfunction according to the German S3-guideline on Dementia 2016 as MMSE 0–9: severe; MMSE 10–19: moderate; MMSE 20–26: mild; MMSE 27–30: no dementia [21].

Microbiome analysis

Total DNA was isolated from frozen stool samples using MagnaPure LC DNA Isolation Kit III (Bacteria, Fungi) (Roche, Mannheim, Germany) according to manufacturer's instructions including mechanic and enzymatic lysis [22]. Hypervariable regions V1-V2 were amplified in a target specific PCR using the primers 27F and R357 (27F-AGAGTTTGATCCTGGCTCAG; R357-CTGCTGCCTYCCGTA) and sequenced with the Illumina MiSeq technique (Illumina, Eindhoven, The Netherlands) [22]. Sequencing was done in cooperation with the Core Facility for Molecular Biology at the Center for Medical Research in Graz.

Gut permeability, inflammation and bacterial translocation

Enzyme linked immunosorbent assays (ELISA) were used to measure fecal and serum calprotectin, fecal and serum zonulin, serum diamine oxidase (Immundiagnostic AG, Bensheim, Germany), soluble (s)CD14 (R&D Systems, Minneapolis, USA), and lipopolysaccharide binding protein (LBP) (Hycult biotech, Uden, The Netherlands). All assays were performed according to manufacturers' instructions. Bacterial products (endotoxin, peptidoglycan and bacterial DNA) were detected in serum using HEK-Blue hTLR4, HEK-Blue hNOD2 and HEK-Blue hTLR9 reporter cells (Invivogen, Toulouse, France), respectively as published previously [23].

Statistical analysis

For microbiome analysis generated FASTQ files were processed for analysis using Qiime2 [24] tools implemented in Galaxy (https://galaxy.medunigraz.at). Denoising (primers removing, quality filtering, correcting errors in marginal sequences, removing chimeric sequences, removing singletons, joining paired-end reads, and dereplication) was done with DADA2 [25]. Taxonomy was assigned based on Silva 132 database release at 99% OTU level, trained using a Naïve Bayes classifier. Sequences were blasted in the NCBI database for further classification [26]. Features with a total sequence count of less than 10 and/or present in less than two patient samples were excluded from analysis. Chloroplast and cyanobacteria filtering were performed to remove contaminants. The resulting mean sequencing depth was 41,631 (range 21,774–53,719) reads per sample. In QIIME2, "feature" is the observational unit and describes a sequence variant/operational taxonomic unit. Analysis was done using the web-based software Calypso 7.14 (http://cgenome.net/calypso/) [27]. For alpha diversity assessment, data was rarefied with a sampling depth of 24,771 reads and Chao1 index, Simpson reciprocal index and Faith phylogenetic diversity were calculated to quantify microbial diversity.

Beta diversity and taxon comparison was done on an unrarefied feature table after total sum scaling and square root transformation. Redundancy analysis based on Bray Curtis dissimilarity was used to compare beta diversity between groups and to identify significant confounders. Differentially abundant taxa were identified with Analysis of Composition of Microbiomes (ANCOM) [28]. This method accounts for compositional constraints and reduces false discovery rates while maintaining high statistical power during the detection of differentially abundant taxa. This test utilizes multiple taxon-to-taxon comparisons and infers differential abundance of a taxon based on the number of significant group comparisons relative to other taxa (W-value). Feature selection was performed using the supervised machine learning tool Linear Discriminant analysis Effect Size (LEfSe) [29]. LEfSe is a tool to discover features by way of class comparison, tests of biological consistency and effect size estimation between two or more microbial communities. All analyses were performed on feature, genus, family, class, order and phylum level. Sequence data is publicly available at the NCBI Sequence Read Archive (SRA accession: PRJNA608281).

All other statistical analyses were performed using SPSS version 25.0 (SPSS Inc., Chicago, Illinois, USA) and R [30] version 3.5.2 (packages: "mice", "ggcorrplot", "psych", "randomForest", "fmsb", "stats", "robustHD") [31–37]. Tests (t-test or Mann-Whitney) were chosen depending on the distribution of the data assessed by Shapiro-Wilk normality test. Spearman rank correlation with Benjamini-Hochberg correction for multiple testing was used to assess strength and direction of linear relationships between variables. All statistical tests were 2-sided, and p-values < 0.05 were considered statistically significant. Data are presented as median and 95% confidence interval unless stated otherwise. Missing values were imputed by multivariate imputation by chained equations (package "mice") [33] based on random forests (package "randomForest") [31]. Univariate and multivariate RDA was performed to find out which variables explain the variance in microbiome composition. VIF values were calculated to account for collinearity between the explanatory variables (package "fmsb"), [37] explanatory variables were standardized for multivariate RDA (package "robustHD") [35]. Network analysis was based on Spearman's rho associations between taxa and converting the pairwise correlations into dissimilarities to ordinate nodes in a two dimensional PCoA plot.

Results

Patient characteristics

We recruited 25 patients with dementia (Alzheimer type and mixed type) and 18 matched controls without cognitive impairment in this prospective controlled cohort study. From 2 dementia patients we were not able to collect enough stool and blood samples to do the intended analyses; therefore, they were excluded from the final analysis. (Fig. 1) Dementia patients had a lower body mass index (BMI) and erythrocyte count as well as lower serum albumin and total protein levels compared to controls. Accordingly, nutritional status according to MNA-SF was significantly worse in dementia patients. Within the dementia group, erythrocyte count ($r = 0.669$, $p = 0.002$) and albumin ($r = 0.707$, $p < 0.002$) showed a significant positive correlation with MMSE and clock drawing test showed a weak positive correlation with albumin ($r = 0.485$, $p = 0.019$). No significant differences were found regarding age, gender, and other routine biochemistry parameters. BMI did not correlate with MMSE or clock drawing test results. Collinearity analysis showed variance inflation factors (VIF) below 2

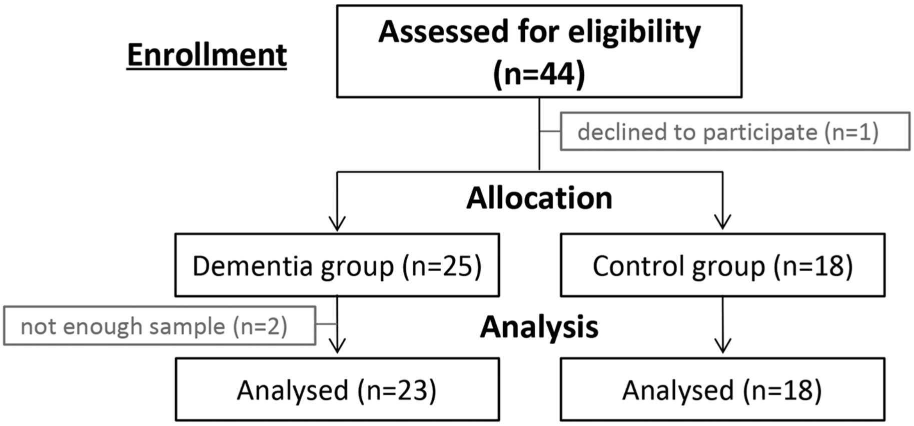

Fig. 1 Patient flow chart

for MMSE, clock-drawing test, BMI, albumin and MNA-SF.

Prescription drug intake was significantly different between dementia patients and controls. Dementia patients took three times more prescription drugs compared to controls. Antidepressants, laxatives, opioids, anti-dementia drugs, sedatives, vitamin D and metamizole were prescribed nearly exclusively in the dementia group, whereas proton pump inhibitors (PPI), antihypertensive drugs, statins, nonsteroidal anti-inflammatory drugs (NSAIDS), paracetamol, antidiabetics, thyroid hormones, calcium and magnesium supplements, anticoagulation and phytotherapeutics were equally prescribed for dementia patients and age matched controls. Laxatives, sedatives, metamizole and paracetamol were usually prescribed as needed, whereas the other drugs were prescribed as fixed dose medication. Patient characteristics are shown in Table 1.

Gut microbiome composition

Alpha diversity using Chao 1 index (Fig. 2a), Simpson reciprocal index (Supplementary figure S1A) or Faith phylogenetic diversity (Supplementary figure S1C) was not significantly different in dementia patients compared to age matched controls. Redundancy Analysis (RDA) showed clear clustering of dementia patients compared to controls (Variance 34.3, $F = 1.31$ $p = 0.003$). (Fig. 2b) Alpha diversity also did not change significantly with

Table 1 Patient characteristics. Data are given as median and 95% confidence interval unless stated otherwise

	Dementia patients (*n* = 23)	Controls (*n* = 18)	*p*-value
Age (years)	88 (73;85)	75 (74;76)	n.s.
Gender (f/m) (n)	15/8	11/7	n.s.
BMI (kg/m2)	24.8 (22.6; 25.9)	28.1 (25.2; 31.0)	$p = 0.028$
MMSE	16 (13;21)	29 (30;30)	$p < 0.0001$
Clock drawing test	3 (0;5)	7 (7;9)	$p < 0.0001$
Number of prescription drugs	9 (6;11)	3 (1;4)	$p < 0.0001$
MNA-SF	10 (9;12)	14 (14;14)	$p < 0.0001$
Leukocytes (10^9/L)	6.6 (6.2;8.3)	6.1 (5.4;7.5)	n.s
Erythrocytes (10^{12}/L)	4.5 (4.0;4.7)	4.7 (4.4; 5.1)	$p = 0.028$
Thrombocytes (10^9/L)	220 (216;248)	216 (205;222)	n.s
Hemoglobin g/dL	13.2 (12.7;14.4)	14.1 (13.3;14.7)	n.s
Creatinine (mg/dL)	0.9 (0.8;1.0)	1.0 (0.9;1.1)	n.s
Bilirubin (mg/dL)	0.6 (0.5;0.9)	0.6 (0.5;0.6)	n.s
Albumin (g/dL)	3.9 (3.7; 4.1)	4.2 (4.1;4.4)	$p = 0.006$
total protein (g/dL)	7.0 (6.8;7.3)	7.5 (7.3;7.6)	$p = 0.014$
CRP (mg/l)	5 (3;11)	2 (1.2; 3.4)	n.s.

BMI body mass index, *MMSE* Mini mental state examination, *MNA-SF* Mini Nutritional Assessment Short Form, *CRP* C reactive protein

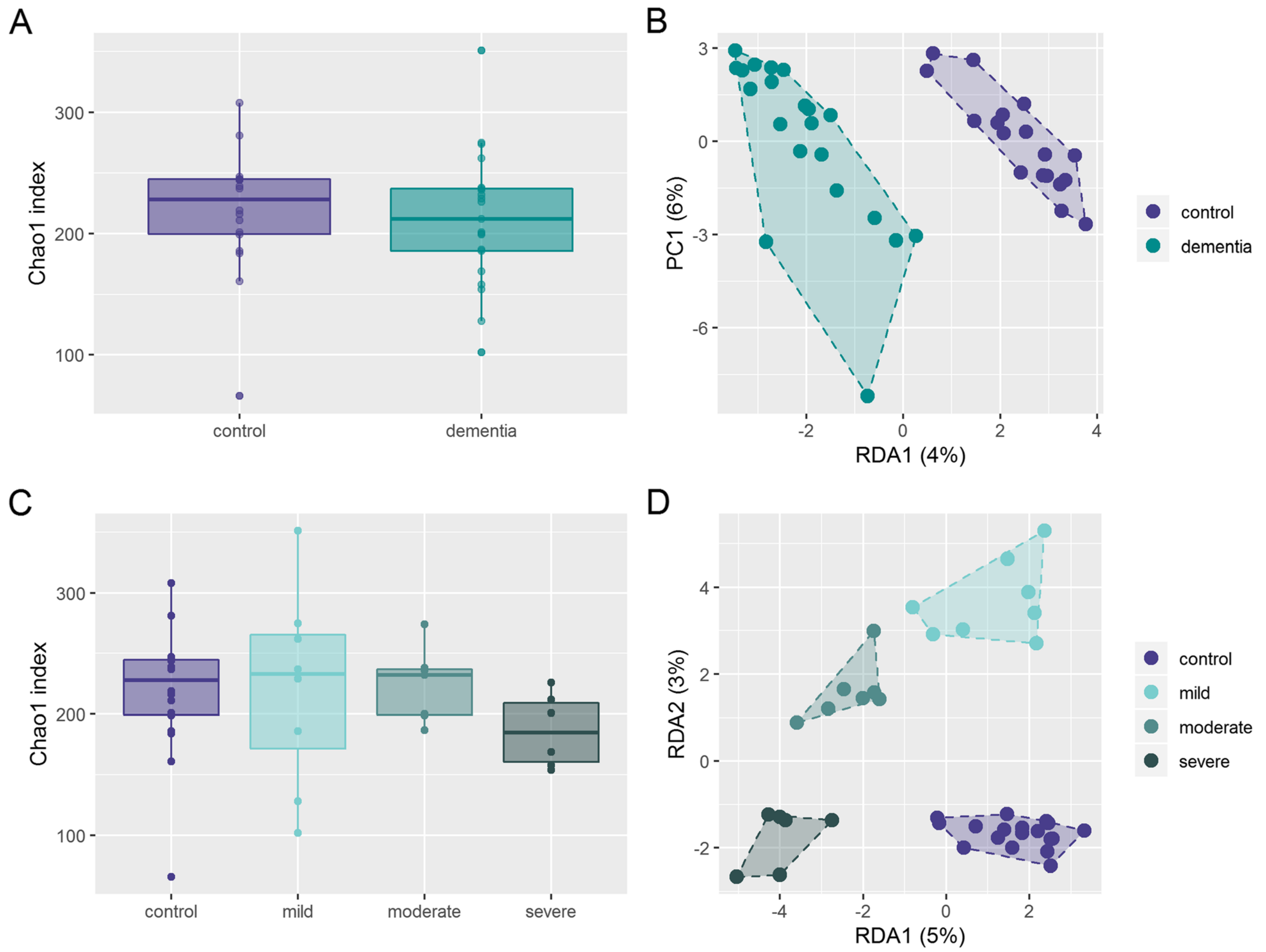

Fig. 2 Differences in alpha and beta diversity in stool microbiome between dementia patients and controls **a** Alpha diversity (Chao1) in dementia patients and controls **b** Beta diversity (RDA) in dementia patients and controls **c** Alpha diversity between controls and different stages of cognitive dysfunction **d** Beta diversity (RDA) in controls and different stages of dementia

increasing degree of dementia. (Fig. 2c and supplementary figure S1B and D) RDA showed clear clustering of different stages of dementia (Variance 94.7, F = 1.2 p = 0.001). (Fig. 2d) Linear discriminant analysis of effect size (LEfSe) identified one family, 5 genera and 7 features to differ between patients with dementia and controls. For example, the features *Clostridium clostridioforme, Anaerostipes hadrus* and *Bacteroides dorei* were associated with dementia; *Lachnospiraceae bacterium MC-35*, another *Lachnospiraceae sp*., and the genus *Lachnospiraceae NK4A136 group* were associated with health. (Fig. 3a) Analysis of Composition of Microbiomes (ANCOM) confirmed that from the taxa identified by LEfSe to discriminate between dementia and control, one uncultured *Lachnospiraceae* feature as well as the genus *Lachnospiraceae NK4A136 group* were significantly less abundant in stool of dementia patients. Additionally, the feature *Eubacterium rectale* was also less abundant in stool of dementia patients (Fig. 3b). When looking at different stages of dementia, LEfSe identified one class, 3 orders, 3 families, 18 genera and 20 features, being associated with severity of cognitive impairment. Most notably, three *Lachnospiraceae* species with the corresponding genus *Lachnospiraceae NK4A136 group* and the genus *Lachnospira* were associated with health; *Faecalibacterium prausnitzii* was associated with mild dementia; moderate dementia was associated with *Lactobacillus amylovorus* and the corresponding higher taxonomic levels (the genus *Lactobacillus*, the family *Lactobacillaceae* and the order *Lactobacillales*). Severe dementia was associated with several potential pathogens (e.g. *Clostridium clostridioforme, Streptococcus salivarius*) (Fig. 4a) From these discriminating taxa, ANCOM analysis identified the feature *C. clostridioforme* and the genus *Eisenbergiella* to increase with severity of cognitive impairment and the family *Lactobacillaceae* to be highest in patients with moderate cognitive impairment. (Fig. 4b).

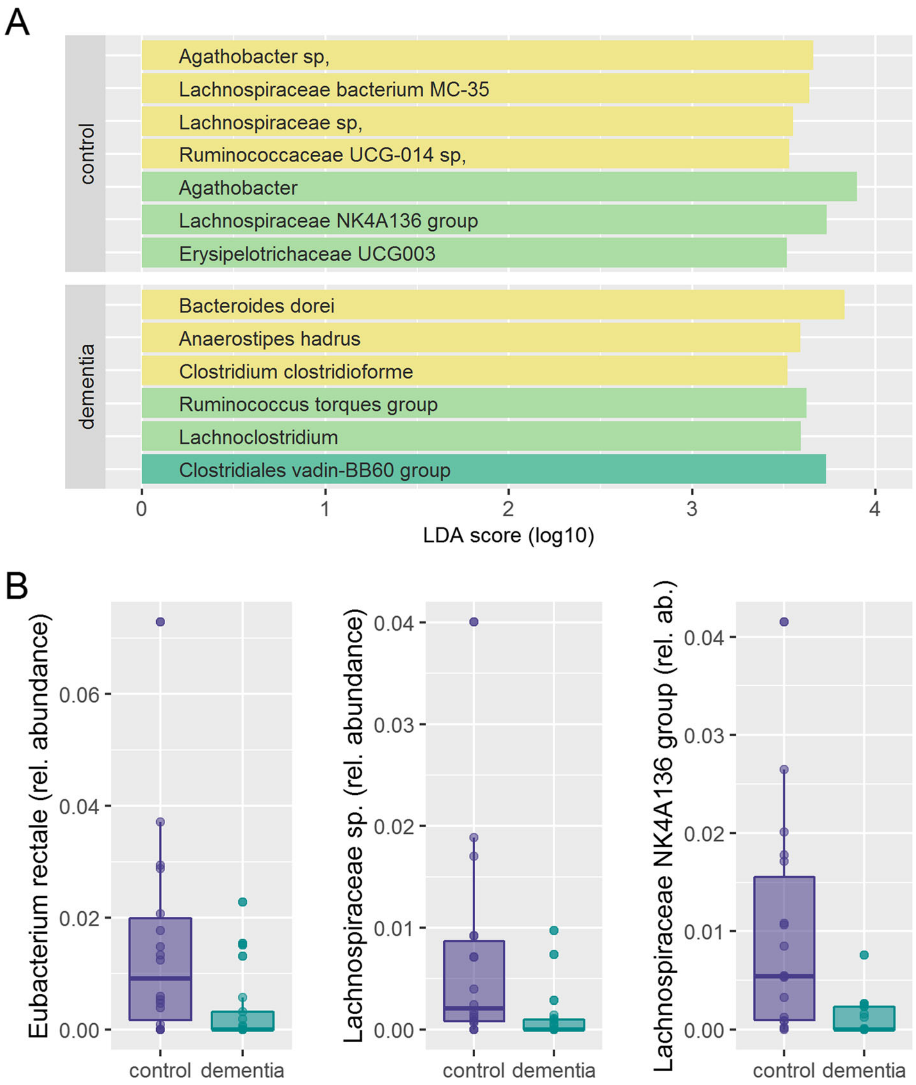

Fig. 3 a Features selected by Linear discriminant analysis Effect Size (LEfSe) to discriminate between dementia patients and controls. **b** Differentially abundant taxa between dementia and controls

Association of drug intake and nutrition on microbiome composition

While some drugs were nearly exclusively prescribed in dementia patients (for details see supplementary table S1), other drugs were equally prescribed between dementia and control subjects. To understand how drug use may influence microbiome composition irrespective of the disease, we studied the effect of drugs that were equally prescribed in dementia patients and healthy controls on diversity measures and taxonomic composition: namely PPI, antihypertensive drugs, statins, thyroid hormones and NSAIDS. Paracetamol, antidiabetics and calcium and magnesium supplements were taken by less than 15% of the cohort and therefore these drugs were not included into the analysis. None of the drugs influenced alpha diversity (Chao1, Simpson, Faith phylogenetic diversity). Statins, but none of the other drugs had a significant impact on beta diversity (RDA, Variance 35.58, F 1.36, p = 0.003). LEfSe identified several features, genera, families, orders and classes being associated with use or non-use of each drug. ANCOM identified several of these taxa as well as some taxa that were not discriminative on LEfSe to be differentially abundant taxa between drug user and non-user. For details see supplementary tables S2–6. Interestingly, PPI use was associated with increased abundance of oral bacteria (e.g. *Streptococcus salivarius*) whereas statin and antihypertensive drug use was associated with increased abundance of bacteria known to produce butyrate (e.g. *Faecalibacterium sp.*).

Since malnutrition was present in 74% of dementia patients but in none of the control persons, microbiome composition in malnourished versus non-malnourished

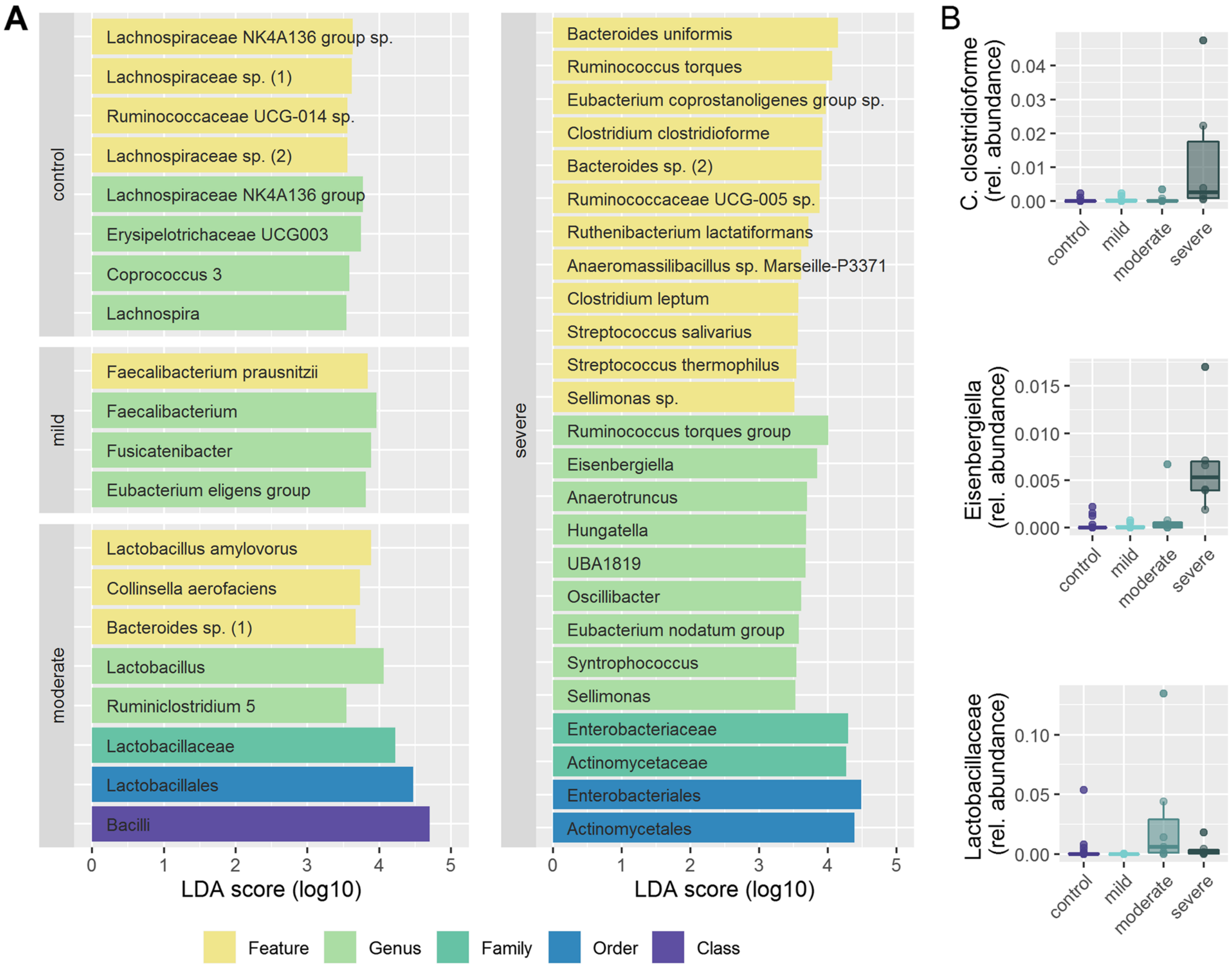

Fig. 4 a Features selected by Linear discriminant analysis Effect Size (LEfSe) to discriminate between dementia different stages of cognitive dysfunction and controls. **b** Differentially abundant taxa between stages of cognitive dysfunction

patients was very similar to the results obtained when comparing dementia versus controls. LEfSe identified the feature *Ruminococcaceae UCG-014 sp* with the corresponding genus *Ruminococcaceae UCG014* and the genus *Lachnospiraceae NK4A136 group* to be associated with normal nutritional state. These taxa were also found to be associated with healthy controls. The genus *Eubacterium hallii group* was associated with dementia. (supplementary table S7) ANCOM confirmed the feature *Ruminococcaceae UCG-014 sp.* and the genus *Lachnospiraceae NK4A136 group* to be differentially abundant between malnutrition and normal nutrition.

Gut barrier dysfunction, inflammation and bacterial translocation

We assessed intestinal permeability by serum diaminooxidase (DAO) and fecal zonulin; inflammation by C-reactive protein, serum lipopolysaccharide binding protein (LBP), soluble CD 14 (sCD14) and fecal calprotectin as well as bacterial translocation by endotoxin, peptidoglycanes and bacterial DNA in serum. Patients with dementia had higher DAO levels and sCD14 levels, indicative for an association with increased gut permeability and increased endotoxin load. (Table 2).

PPI use was associated with significantly increased faecal calprotectin levels (PPI use: 92.5 ng/ml (50.2; 120.5); PPI non-use: 28.1 ng/ml (20.8; 47.9); $p = 0.008$). Antihypertensive use was associated with significantly increased CRP levels (antihypertensive use: 6 mg/dl (3; 11); antihypertensive non-use 1.3 mg/dl (1;4); $p = 0.016$), suggesting complex relations between disease, drug use and inflammation.

Multivariate and network analysis of potential factors influencing microbiome composition in dementia

To understand the main drivers of dysbiosis in dementia we further performed univariate and multivariate RDA to assess the association of clinical variables and

Table 2 Biomarker for gut barrier dysfunction, inflammation and bacterial translocation, Data are shown as median and 95% confidence interval

	Dementia patients (*n* = 23)	Controls (*n* = 18)	*p*-value
Serum diaminooxidase (U/ml)	20.8 (9.7;29)	11.2 (8.4; 13.8)	0.025
Fecal zonulin (ng/ml)	33.8 (31.2; 57)	55.1 (40.8; 76.7)	n.s
C-reactive protein (mg/L)	5 (4; 11)	2 (1.2;3.4)	n.s
Serum lipopolysaccharide binding protein (μg/ml)	17.9 (16.1; 18.6)	20.0 (14.6; 21.3)	n.s
Soluble CD 14 (μg/ml)	2.4 (1.9; 3.1)	1.8 (1.7; 2.1)	0.022
Fecal calprotectin (ng/ml)	31.5 (26.6; 85.8)	49.0 (18.2; 66.3)	n.s
Endotoxin (EU/ml)	0.26 (0.0; 0.33)	0.25 (0.09; 0.53)	n.s
Peptidoglycan[a] (ng/mL)	0.96 (0.26; 1.66)	0.42 (0.30;1.05)	n.s.
Bacterial DNA (μM)	0.06 (0.00;1.46)	0.7 (0.0; 1.29)	n.s

[a] peptidoglycan was only measurable in 12% of the samples, therefore median and confidence interval only for the positive samples are shown. *CD* cluster of differentiation, *EU* endotoxin units

biomarkers with microbiome composition. RDA showed that BMI, albumin, total protein, sCD14, statins, NSAIDs, number of drugs, MNA-SF, MMSE, clock-drawing test, sex, number of drugs were explanatory variables for microbiome composition in controls compared to dementia and between different stages of cognitive dysfunction ($p < 0.1$) (supplementary table S8). To the final multivariate RDA model explanatory variables with $p < 0.1$ in the univariate analysis were included and variables with VIF > 2 in multicollinearity analysis were excluded. (Table 3) In the multivariate model BMI and statin use were the remaining significant explanatory variables for differences in microbiome composition between dementia and control groups and between the groups of dementia severity in the dementia group only. (Table 3) Network analysis also illustrates the overlap between factors influencing microbiome composition: Genera associated with dementia (red) overlap with genera associated with no statin intake (yellow) and BMI (green), whereas genera associated with health (blue) overlap with genera associated with statin intake (purple). (Fig. 5a) When performing network analysis in the subgroup of dementia patients only, the overlaps are less clear, but again genera associated with severe dementia (red) overlap with genera associated with no statin intake (yellow) and genera associated with mild dementia (blue) overlap with genera associated with statin intake (purple). The association with BMI is less pronounced in the dementia subgroup. (Fig. 5b).

Discussion

Our cross-sectional controlled pilot cohort study shows that dementia is associated with changes in microbiome composition including a reduction in bacteria known to produce short chain fatty acids (SCFA) and increased biomarkers of gut permeability and inflammation. Furthermore, we could show that both malnutrition and drug intake are factors associated with microbiome composition in dementia. This study therefore supports the concept of a disrupted gut-brain axis in dementia.

The concept of a disrupted gut-brain axis in dementia has recently emerged. Several animal studies show that induction of dysbiosis by antibiotics, irradiation or

Table 3 Multivariate RDA to identify the most important explanatory variables for microbiome composition changes

Variable	Control versus Dementia	Severity of dementia
BMI	Variance = 33.18 F = 1.29 ***P* = 0.006**	Variance = 33.18 F = 1.29 ***P* = 0.008**
Total protein	Variance = 29.19 F = 1.14 *P* = 0.067	Variance = 29.19 F = 1.14 *P* = 0.070
soluble CD14	Variance = 29.06 F = 1.13 *P* = 0.072	Variance = 29.06 F = 1.13 *P* = 0.079
Statins	Variance = 32.06 F = 1.25 ***P* = 0.009**	Variance = 32.06 F = 1.25 ***P* = 0.014**
Clock-drawing test	Variance = 25.89 F = 1.01 *P* = 0.376	Variance = 25.89 F = 1.01 *P* = 0.374
Age	Variance = 25.88 F = 1.01 *P* = 0.427	Variance = 25.88 F = 1.01 *P* = 0.409
Sex	Variance = 27.63 F = 1.08 *P* = 0.137	Variance = 27.63 F = 1.08 *P* = 0.154
NSAIDS	Variance = 28.17 F = 1.10 *P* = 0.098	Variance = 28.17 F = 1.10 *P* = 0.107

BMI body mass index, *NSAIDS* non-steroidal anti-inflammatory drugs

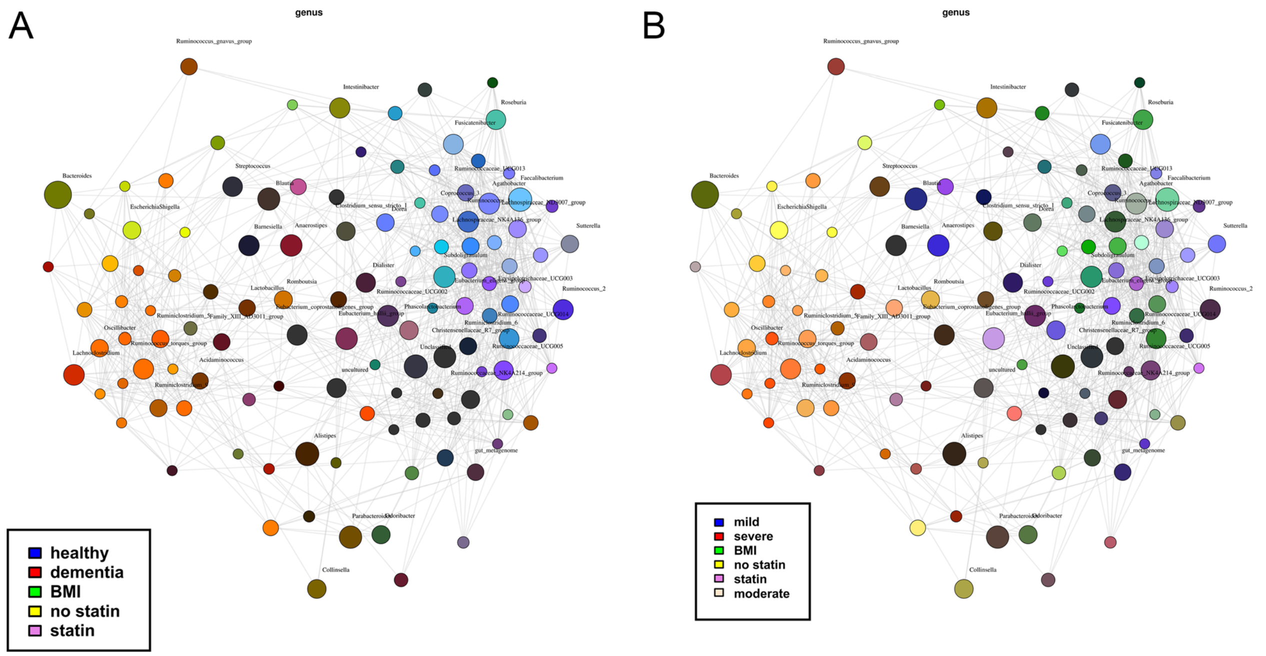

Fig. 5 Network analysis to identify associations between bacteria and selected host variables. Taxa and explanatory variables are represented as nodes, taxa abundance as node size, and edges represent positive associations. Nodes (genera) are coloured based on their association with selected host variables (dementia/health, dementia stages, statin use or non-use, BMI). **a** whole cohort ($n = 41$), **b** dementia patients ($n = 21$)

germ-free conditions negatively impact on cognitive function and plaque deposition as recently reviewed by Ticinesi et al. [38] Recently, in patients with dementia a reduction in diversity of the microbiome has been described, however data on taxonomic microbiome composition are varying between different studies from different geographical locations [9, 10, 39]. We describe altered beta diversity and distinct taxonomic changes in dementia in a European cohort. Alpha diversity data in dementia so far are conflicting, since a lower alpha diversity has been observed in the study from the USA [9], whereas in the Japanese study a lower alpha diversity was observed in the control group [10] but we found unchanged alpha diversity of the gut microbiome between European dementia patients and healthy age matched controls. Microbial diversity depends on many factors, especially in elderly, where the microbiome is likely to be less stable [40]. Elderly people are more often exposed to microbial community-altering events, such as infections and concomitant antibiotic use, polypharmacy or hospital stays. Therefore, elderly controls may not be healthy in a strict sense and selection of the control cohorts may account for the different findings in different studies. Differences in analysis techniques [41] and also in geographic location [42] may be further factors that impact on diversity and composition of the gut microbiome.

When looking at taxonomic differences, abundance of *Eubacterium rectale*, an uncultured *Lachnospiraceae sp.* and *Lachnospiraceae NK4A136 group* was lower in dementia patients compared to controls. LEfSe also identified the family *Lachospiraceae* with its genus *Lachnospiraceae NK4A136* and several *Lachnospiraceae* species to be associated with health. *Eubacterium rectale* is a well-known butyrate producing bacterium [43] and has already previously been associated with cognitive decline [44]. Members of the *Lachnospiraceae* family have been linked to obesity on the one hand and protection from colon cancer in humans on the other hand, likely due to the association of many species within the group with the production of butyric acid, a SCFA that is important for host epithelial cell growth and integrity [45]. Mild dementia was also associated with another butyate producer – *F. prausnitzii* [46]. Increasing the number of butyrate producing bacteria in dementia may therefore be a promising therapeutic approach, since SCFA such as butyrate are critically involved in microglia maturation and function [2, 3]. Data from animal and human pilot studies support this concept. A dietary intervention with bilberry anthocyanin extract was able to increase *Lachnospiraceae NK4A136 group* abundance and improve gut barrier function in ageing rats [47]. An exploratory pilot study in patients with dementia showed that a multispecies probiotic can increase the abundance of butyrate producing bacterial strains [48]. We also found that the abundance of *C. clostridioforme* and the genus *Eisenbergiella* increased with increasing cognitive impairment. *C. clostridioforme* has mainly been described as a human pathogen [49] but has also been described to be associated with vegetarian diet [50]. The genus *Eisenbergiella* was recently found to be increased in long lived adults [51]. Therefore these findings are difficult to interpret in the context of cognitive dysfunction. The family *Lactobacillaceae* was differentially abundant in different stages of dementia, with the highest abundance in moderate dementia and a lower abundance in mild dementia and severe dementia. LEfSe also revealed the bacterium *L. amylovorus* and the corresponding genus *Lactobacillus*, the family *Lactobacillaceae* and the order *Lactobacillales* to be associated with moderate dementia. This finding is also difficult to interpret, since *Lactobacillus sp.* in general were already more than 100 years ago associated with longevity by the Nobel prize winner Elie Metchnikow in 1907 [52] and several studies using different *Lactobacillus sp.* have been conducted with varying success in neurodegenerative diseases [53]. *Lactobacillus amylovorus* has been described as a novel probiotic strain that is able to reduce ammonia levels and may therefore be associated with cognitive function [54].

Our study also shows that dementia is not only associated with dysbiosis but also associated with markers of increased gut permeability (DAO) and markers of inflammation (sCD14). Ageing itself has been associated with an increase in gut permeability, mucosal inflammation and bacterial translocation – often termed as "inflammageing" [2]. Increased calprotectin levels in stool as a sign of intestinal inflammation have been observed in a pilot study [55]. Another study in dementia showed a decrease in previously elevated zonulin levels after probiotic treatment as a possible hint towards a causal link between dysbiosis and gut permeability in dementia [48]. Although we did not find any differences in stool zonulin and calprotectin levels, we found an increase in DAO levels which has been proven to be a valuable serum biomarker of gut barrier dysfunction [56–59]. Also, Ginko biloba, a commonly used phytotherapeutic drug in dementia, was able to reduce DAO levels in an animal model of alcoholic liver disease, indicating both the validity of DAO as a permeability biomarker and that gut hyperpermeability may be a modifiable and relevant therapeutic target [60]. We furthermore found elevated sCD14 levels in dementia as a marker of endotoxemia and inflammation. Recent in vitro data suggest that the altered stool microbiome composition in dementia directly modulates intestinal epithelial homeostasis via the anti-inflammatory P-glycoprotein pathway [11].

In our cohort, dementia patients, although not different regarding age and gender from our controls, received 3 times more prescription drugs. Although some of these drugs were only prescribed on demand, this finding is still striking. The known consequences of polypharmacy are among others, cognitive impairment, a higher risk of falls and non-compliance, but interventions to reduce polypharmacy are difficult [61, 62]. Drug-microbiome interactions are increasingly recognized. A population based deep sequencing study revealed, that proton pump inhibitors (PPI) were associated with the most profound microbiome changes, followed by statins, antibiotics, laxatives and beta blockers [17]. It has been shown experimentally that not only classic antimicrobials but also many other human-targeted drugs have an extensive impact on human gut bacteria [15]. We recently showed that PPI are one of the main drivers of dysbiosis in liver cirrhosis [63, 64]. We therefore assessed the association of prescription drugs with gut microbiome composition. As expected, effects on overall community structure (alpha and beta diversity) were small. Each drug class was associated with distinct associations throughout different taxonomic levels between users and non-users. PPI use was associated with higher abundance of oral bacteria in the stool and statins and antihypertensive use was associated with an increase in SCFA producing bacteria. Due to the small sample size, the results have to be interpreted with caution and can only serve as pilot data that need to be explored in larger cohorts. Additionally, we assessed the impact of drug intake on markers of gut permeability, bacterial translocation and inflammation. We found that PPI use was associated with increased intestinal inflammation. This has been previously described in the context of other diseases [65–67] and we have recently linked dysbiosis, gut permeability and intestinal inflammation to adverse outcome in patients with liver cirrhosis who use PPI [63]. Antihypertensive use was associated with slightly, but significantly elevated CRP levels, which is most likely due to the underlying disease and not to the drug itself, since arterial hypertension is associated with elevated CRP levels [68] and therefore validates the relevance of our findings although sample size is small.

Malnutrition is common in dementia and nutrition care is an integral part of dementia care [69]. Although all patients in our dementia cohort were treated according to nutritional support standards that include oral nutritional supplements in patients with MNA-SF < 9, MNA-SF and laboratory parameters showed that more than three quarter of the dementia patients in our study were malnourished. It is therefore impossible from this cross-sectional pilot study to distinguish if malnutrition or dementia are the starting point of dysbiosis. This could only be answered by longitudinal studies. In general, malnutrition has been associated with differences in microbiome composition, such as loss of bifidobacteria, however, most studies were performed in malnourished children and not in elderly people [70].

In order to identify the drivers of dysbiosis we performed multivariate analyses with all variables and excluded variables that showed high collinearity to understand the driver of dysbiosis. We found that BMI and statin use were the strongest influencing factors, underpinning the notion that malnutrition and prescription drug use drive microbiome composition in dementia. Also network analysis supports the close association of these factors. However, due to the small sample size, the results of our multivariate analysis have to be interpreted with caution and can be seen as hypothesis generation only. The results need to be confirmed in larger studies.

Our study has some limitations: First, the single center approach and the sample size limit the generalizability of the data. A combination of all studies on gut microbiome in dementia would be desirable, however, due to the lack of standards in sequencing techniques this would not be technically feasible. Second, we could not perform the gold standard analysis of gut permeability – the differential sugar absorption test – because of the cognitive impairment of our patients, who were not able to follow the instructions of the test. We overcame this by using a panel of serum and stool markers that do not require compliance with test instructions for the participants. And third, this study only provides cross-sectional data and can therefore not answer any questions regarding causality or cause-effect relationship between cognitive dysfunction dysbiosis and malnutrition. A longitudinal study is in planning.

Conclusion

In summary this study provides evidence that structural changes in microbiome composition in dementia are associated with malnutrition and prescription drug use and that biomarkers of gut permeability are increased in dementia. Further studies to move from associations to causality in understanding the gut-brain axis in dementia are necessary. Increasing butyrate producing bacteria and targeting malnutrition seems to be promising therapeutic approaches to treat dementia related dysbiosis. The effect of microbiome modulating strategies on cognitive function needs to be addressed in future studies.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12877-020-01644-2.

Additional file 1: Figure S1. Alpha diversity in stool microbiome between dementia patients and controls A: Alpha diversity (Faith PD) in dementia patients and controls B: Alpha diversity (Faith PD) in controls and different stages of cognitive dysfunction C: Alpha diversity (Simpson) between dementia patients a D: Alpha diversity (Simpson) in controls and different stages of cognitive dysfunction. **Table S1.** Drug intake in dementia patients and controls. **Table S2.** Features selected by LEfSe to discriminate between PPI users and non-users. **Table S3.** Features selected by LEfSe to discriminate between statin users and non-users. **Table S4.** Features selected by LEfSe to discriminate between antihypertensive users and non-users. **Table S5.** Features selected by LEfSe to discriminate between NSAID users and non-users. **Table S6.** Features selected by LEfSe to discriminate between thyroid hormone users and non-users. **Table S7.** Features selected by LEfSe to discriminate between malnourished and non-malnourished participants. **Table S8.** Redundancy analysis with explanatory variables of microbiome composition changes.

Abbreviations

AD: Alzheimer's disease; ANCOM: Analysis of Composition of Microbiomes; BMI: Body mass index; CRP: C reactive protein; DAO: Diamino oxidase; LEfSe: Linear Discriminant analysis Effect Size; LBP: Lipopolysaccharide binding protein; MMSE: Mini Mental State Examination; MNA-SF: Mini Nutritional Assessment Short Form; NSAIDS: Nonsteroidal anti-inflammatory drugs; PPI: Proton pump inhibitors; RDA: Redundancy Analysis; SCFA: Short chain fatty acids; sCD14: Soluble CD14 levels; VIF: Variance inflation factor

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Authors' contributions

VS planned the study, acquired funding, performed analyses, analyzed data and wrote the manuscript; LE performed analyses, analyzed data and wrote the manuscript; IK analyzed data and wrote manuscript, NF collected data, performed analyses, and critically reviewed the manuscript; BL performed analyses and critically reviewed the manuscript, GP, NF and MS recruited patients, collected data and critically reviewed the manuscript, RS and WS critically reviewed the manuscript, AH analyzed data and wrote the manuscript. All authors have read and approved the manuscript

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Availability of data and materials

Sequence data is publicly available at the NCBI Sequence Read Archive (SRA accession: PRJNA608281). The remaining datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study (29–420 ex 16/17) was approved by the ethics committee Ethic Committee of the Medical University of Graz (IRB00002556). The study was performed according to the Declaration of Helsinki and Good Clinical Practise guidelines. Written informed consent was obtained before any study specific procedure was performed from participants or their legal representatives (in case patients were not able to give written consent any more due to the severity of cognitive dysfunction). This procedure was approved by the ethics committee.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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