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Thesis presented *to Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Biomedical Engineering

by

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I dedicate this thesis to my father,
Rogério Apolinário, whose wisdom, guidance,
and unwavering support I will forever treasure.

Resumo

A Doença de Alzheimer (DA) é uma doença neurodegenerativa progressiva caracterizada pelo declínio cognitivo, frequentemente associada ao acúmulo de placas amiloides e emaranhados de tau no cérebro. Estudos recentes sugerem que o microbioma intestinal pode desempenhar um papel na patogénese da DA, com a disbiose intestinal a contribuir potencialmente para a neuroinflamação através do eixo intestino-cérebro. Esta tese explora o potencial discriminatório da composição do microbioma intestinal entre pacientes com DA e controlos saudáveis (CS), com o objetivo de identificar biomarcadores baseados no microbioma para o diagnóstico precoce da DA.

A análise clínica e demográfica incluiu idade, sexo, presença do alelo apolipoproteína-E (ApoE4) e índice de massa corporal, com diferenças significativas de idade e frequência do alelo ApoE4 observadas entre os grupos DA e CS. A análise do microbioma examinou índices de diversidade alfa (Shannon e Simpson) e composição taxonómica, revelando alterações microbianas específicas, particularmente nos géneros *Escherichia-Shigella* e *Adlercreutzia*, que podem estar associadas à patologia da DA.

Modelos de aprendizagem computacional foram usados para avaliar o poder preditivo das características do microbioma para a DA, incorporando variáveis clínicas. Os modelos foram otimizados para precisão e robustez através da seleção de características e redução de dimensionalidade (fusão de dados). O modelo com melhor desempenho, avaliado através de Leave-One-Out Cross-Validation (LOOCV), alcançou uma AUC de 0.88 e uma precisão de 0.89, refletindo uma melhoria em relação ao modelo do estudo de referência, que obteve uma AUC máxima de 0.83. Estes resultados destacam a capacidade discriminatória aprimorada do modelo.

Estes resultados sublinham o potencial do microbioma intestinal na descoberta de biomarcadores para a DA, com as alterações microbianas identificadas a oferecerem insights para futuras investigações diagnósticas e terapêuticas. Recomenda-se a realização de estudos adicionais para refinar estes modelos preditivos e explorar a influência do microbioma na progressão da DA, avançando em direção a uma ferramenta de diagnóstico baseada no microbioma que seja fiável e acessível.

Palavras-chave: Doença de Alzheimer, microbioma intestinal, dados taxonómicos, aprendizagem computacional, predição;

Abstract

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder marked by cognitive decline, commonly linked to the accumulation of amyloid plaques and tau tangles in the brain. Recent studies suggest that gut microbiota plays a role in AD pathogenesis, with gut dysbiosis potentially contributing to neuroinflammation through the gut-brain axis. This thesis explores the discriminatory potential of gut microbiota composition between AD patients and healthy controls (HC), aiming to identify microbiome-based biomarkers for early AD diagnosis.

The clinical and demographic analysis included age, sex, apolipoprotein-E (ApoE4) allele presence, and body mass index (BMI), with significant age differences and ApoE4 allele frequency observed between AD and HC groups. Microbiome analysis examined alpha diversity indices (Shannon and Simpson) and taxonomic composition, uncovering specific microbial shifts, particularly within *Escherichia-Shigella* and *Adlercreutzia*, that may be associated with AD pathology.

Machine learning models were used to assess the predictive power of microbiome features for AD, incorporating clinical variables. The models were optimized for accuracy and robustness through feature selection and dimensionality reduction (data fusion). The best-performing model, evaluated through Leave-One-Out Cross-Validation (LOOCV), achieved an AUC of 0.88 and an accuracy of 0.89, reflecting an improvement over the model in the referenced study, which attained a maximum AUC of 0.83. These scores underscore the model's enhanced discriminatory capacity.

These findings underscore the gut microbiota's potential in AD biomarker discovery, with identified microbial alterations offering insights for future diagnostic and therapeutic research. Further studies are recommended to refine these predictive models and explore the microbiome's influence on AD progression, advancing towards a reliable and accessible microbiome-based diagnostic tool.

Keywords: Alzheimer's disease, gut microbiota, taxonomic data, machine learning, prediction;

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I would like to begin by thanking my supervisor, Prof. Pedro Rodrigues, for his guidance and support throughout this journey and my co-supervisor, Prof. Patrícia Batista, with whom I have worked beside for the past two years and has always pushed me to become a better researcher.

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To my mother and brother, thank you for your unwavering support and encouragement during this time.

And, finally, a heartfelt thanks to Sardas for being a pillar in my life with whom I always know I can count on.

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List of Abbreviations and Symbols

Aβ – Beta Amyloid

AD – Alzheimer's Disease

ApoE4 – Apolipoprotein-E4

AUC – Area-Under-the-Curve

AUROC – Area Under the Receiver Operating Characteristic Curve

CSF – Cerebrospinal Fluid

EEG – Electroencephalogram

fNIRS – Functional Near-Infrared Spectroscopy

FP – False Positives

G-Mean – Geometric Mean

HC – Healthy Control

LogReg – LogisticRegression

LOOCV – Leave-One-Out Cross-Validation

LPS – Lipopolysaccharides

MCI – Mild Cognitive Impairment

ML – Machine Learning

MLP – Multi-Layer Perceptron

MMSE – Mini-Mental State Examination

MoCA – Montreal Cognitive Assessment

MRI – Magnetic Resonance Imaging

NFTs – Neurofibrillary Tangles

NMDA – N-Methyl-D-Aspartate

PCA – Principal Components Analysis

PET – Positron Emission Tomography

RF – RandomForestClassifier

SVMs – Support Vector Machines

TN – True Negatives

TP – True Positives

1. Introduction

Alzheimer's Disease (AD) is a chronic neurodegenerative disease that affects populations worldwide, with a particular incidence in the elderly population. This pathology manifests as a gradual and irreversible decline in cognitive abilities, especially memory, language, and critical thinking. The exact causes of AD are still not fully understood, and the difficulty of early diagnosis compromises intervention. Therefore, due to the demographic situation and the incidence of the disease at different stages, research into the early detection of the pathology is urgent to intervene, slow the progression of the disease, improve patient's quality of life, and reduce healthcare costs.

Currently, there are a variety of tests and assessments to identify cognitive impairment and evaluate the likelihood of developing AD, including neuropsychological tests, brain imaging, and genetic tests. Recent studies suggest that gut microbiota composition may be closely linked to neurodegenerative processes involved in AD. This connection is hypothesized to occur through the gut-brain axis, a bidirectional communication pathway that allows the gut microbiota to influence brain health via neural, immune, and endocrine pathways. Alterations in gut microbiota, known as dysbiosis, have been associated with systemic inflammation and neuroinflammation, both potential contributors to AD progression.

In this context, exploring the gut microbiota as a potential biomarker for early AD detection has gained attention. Identifying specific microbial profiles that differentiate AD patients from healthy individuals could pave the way for innovative diagnostic tools that are both accessible and non-invasive. Furthermore, integrating machine learning (ML) techniques with microbiome data analysis could enhance predictive accuracy, enabling more reliable identification of at-risk individuals. Such advancements hold promise for developing early intervention strategies that may slow cognitive decline, improve the quality of life for patients, and reduce the burden on healthcare systems.

1.1. Alzheimer's Disease

AD is a neurodegenerative disease mainly characterized by progressive cognitive decline, including loss of short-term memory and impairments in speech, visuospatial processing, and executive functions (mental processes such as attention control, cognitive flexibility, cognitive inhibition) (Knopman et al., 2021) leading to dependence for essential life

functions and eventually premature death (Mayeux & Stern, 2012). AD is fundamentally characterized by extensive brain alterations, particularly neuroinflammation and the progressive degeneration of neural structures (C. Wang et al., 2023). This is caused by neuronal damage due to the accumulation of abnormal proteins in the temporal lobe of the brain and hippocampus (Afzal et al., 2021).

The prevalence of AD is constantly increasing alongside life expectancy. As of 2019, it is estimated that 5.8 million Americans have AD, with 96% of them belonging to the elderly population (people aged 65 years and older), and this number is set to increase to at least 13 million in the US by 2050, which translates to a new case every 33 seconds ("2019 Alzheimer's Disease Facts and Figures," 2019). Overall, AD is responsible for around 70% of dementia cases (Reitz et al., 2011), which had a $56.000 annual cost per patient in 2015 and an overall 360 billion dollars in total payments (Sakshi Mirzapure, 2022). This, alongside its social impacts, highlights the need for new therapeutic strategies to detect AD in its early stages.

AD is marked by the accumulation of beta-amyloid (Aβ)-containing extracellular plaques and tau-containing neurofibrillary tangles (NFTs), both of which contribute to neuroinflammatory processes and neuronal damage (G. Zhang et al., 2022). The widespread distribution of these amyloid plaques and NFTs is accompanied by astrogliosis, synaptotoxicity, neuronal loss, and vascular alterations (Cao et al., 2024). Aβ is a peptide derived from the amyloid precursor protein as a byproduct of its cleavage. It is produced by all cells, but particularly at high levels during synaptic activity (G. Chen et al., 2017). Aβ serves several physiological roles, including neuroprotection during aging, antimicrobial activity, regulation of the blood-brain barrier, and enhancing recovery from post-traumatic brain injury (Volicer, 2020). Nevertheless, Aβ can aggregate through complex processes to form small oligomers and fibrils. Oligomers are believed to be responsible for synaptic dysfunction and memory deficits in AD patients by inhibiting long-term potentiation and damaging dendritic spines (G. Chen et al., 2017; Araki & Kametani, 2022). Fibrils are insoluble protein aggregates composed of repeating β-sheet structures, that disrupt the structural integrity of neuronal networks, induce oxidative stress leading to neuronal apoptosis, and form amyloid plaques that trigger chronic inflammatory responses (Knopman et al., 2021).

On the other hand, tau is a microtubule-associated protein found in axons, usually stabilizes microtubules to support intracellular transport. However, tau undergoes abnormal hyperphosphorylation and misfolding in AD, accumulating as NFTs within neurons (Guo et al., 2017). These tangles impair intracellular transport and communication, ultimately causing

neuronal death (d'Errico & Meyer-Luehmann, 2020). So, these pathological features contribute to the cognitive decline and neurodegeneration of AD.

Genetic predispositions also play a significant role in AD, with particular attention to the apolipoprotein-E4 (ApoE4) gene, which is a major genetic risk factor for the condition. The ApoE4 gene encodes apolipoprotein E, a protein involved in lipid metabolism and neuronal repair processes (Uddin et al., 2019). Of the three alleles (ApoE2, ApoE3,ApoE4), the ApoE4 allele is most strongly associated with AD. Individuals carrying one copy of this allelo have a threefold increased risk, while those with two copies face up to a tenfold risk compared to non-carriers (Emrani et al., 2020; Safieh et al., 2019).This allele is believed to contribute to AD pathogenesis by disrupting Aβ clearance and promoting aggregation, impairing lipid transport and mitochondrial function, and exacerbating neuroinflammation. Moreover, ApoE4 carriers tend to present with earlier disease onset, making it a critical factor for stratifying risk in clinical and research settings (Safieh et al., 2019).

1.2. AD diagnosis and treatment

AD is a progressive, irreversible neurodegenerative disease impacting cognition, function, and behavior. AD follows a progressive disease continuum that extends from an asymptomatic phase with biomarker evidence of AD (preclinical AD) through minor cognitive (mild cognitive impairment [MCI]) and neurobehavioral (mild behavioral impairment [MBI]) changes to, ultimately, AD dementia (Jack et al., 2018).

The clinical diagnosis of AD is fundamentally based on assessing cognitive, functional, and behavioral symptoms (Apostolova, 2016). Memory impairment, particularly of short-term memory, is often the initial and most prominent symptom, followed by declines in episodic, working, and semantic memory as the disease advances. Other cognitive domains, such as language and visuospatial skills, also deteriorate progressively (Berente et al., 2022). Cognitive assessments like the Mini-Mental State Examination (MMSE) and the Montreal Cognitive Assessment (MoCA) are essential tools in this process. The MMSE is widely used to screen for cognitive impairment by evaluating a broad range of cognitive domains, including orientation, registration, attention, recall, and language (Pavel, 2020). However, it is less sensitive to detecting MCI in the early stages of AD (Pavel, 2020). The MoCA, in contrast, is more sensitive in detecting mild impairments by including more complex tasks assessing executive function, attention, and visuospatial abilities (Cersonsky et al., 2022). Studies have shown that the MoCA

Total Score and MoCA Memory Index Score are useful for predicting MCI conversion to AD, with predictive accuracy as high as 91% when both scores fall below established thresholds (Dautzenberg et al., 2020). This makes the MoCA particularly effective in identifying early-stage cognitive changes that may not be apparent on the MMSE (Cersonsky et al., 2022).

Behavioral symptoms, including irritability, agitation, sundowning, psychosis, and anxiety, typically emerge early and intensify over time, imposing significant psychological and physical burdens on caregivers (Apostolova, 2016). Neurological symptoms, manifesting in the later stages, often precipitate severe complications like aspiration, malnutrition, infections, and thrombosis, leading to premature mortality (Zvěřová, 2019).

Identifying Alzheimer's-related symptoms and pathologies early and accurately is essential to advancing our understanding and management of this complex disease, and recent advancements in diagnostic methodologies now play a pivotal role. Thus, in 2019, the National Institute on Aging and the Alzheimer's Association expanded the diagnostic criteria for AD to incorporate supportive biomarker tests, quantifying disease-specific biological factors (Márquez & Yassa, 2019). These biomarkers fall into two primary categories: fluid biomarkers and neuroimaging biomarkers. Fluid biomarkers involve the analysis of cerebrospinal fluid (CSF) to measure levels of Aβ and tau proteins, where decreased CSF Aβ suggests increased plaque formation and elevated CSF tau indicates enhanced neurodegeneration (Teipel et al., 2022). Blood-based biomarkers, such as plasma neurofilament light chain, offer a less invasive alternative for assessing Aβ and phosphorylated tau levels.

Neuroimaging biomarkers enable the visualization and quantification of neurodegenerative and molecular changes by providing brain structural and functional details (Teipel et al., 2022). Magnetic resonance imaging (MRI) utilizes blood-oxygenation-level-dependent contrast, which correlates with neuronal activity (Göttler et al., 2019). Positron emission tomography (PET) assesses neuropathological markers such as fibrillar Aβ (Márquez & Yassa, 2019). Electroencephalogram (EEG) detects electrical brain activity changes, such as altered delta and theta bands linked to AD. Still, its lower spatial resolution limits detailed imaging compared to MRI and PET (Cicalese et al., 2020). Recently, a new technology enhanced EEG with functional near-infrared spectroscopy (fNIRS), which monitors brain oxygenation, providing better spatial resolution and improving diagnostic accuracy by capturing electrical activity and hemodynamic responses (Cicalese et al., 2020).

All these imaging modalities have demonstrated high efficacy in detecting and monitoring AD progression, as shown in Table 1.1.

Table 1.1 Promising biomarkers for Alzheimer's Disease diagnosis in a mild cognitive impairment stage are evaluated using area-under-the-curve (AUC) and accuracy score.

		AUC	Accuracy	Advantages	Disadvantages	Current state	Ref
Neuroimaging	PET	0.93	87%	High efficacy in detecting and monitoring AD progression, non-invasive	Expensive; exposure to radioactive tracers	Clinically validated	(Palmqvist et al., 2015)
	MRI	0.95	94%	High Sensitivity, specificity, non-invasive	Expensive, less specific to molecular changes	Clinically validated	(X. Chen et al., 2022)
	EEG	0.97	93%	Widely available, Non-invasive	Lower spatial resolution, less effective alone	Clinically validated	(Ge et al., 2020)
	EEG - fNIRS	0.87	79%	Provides complementary insights into brain function and activity	It is more complex and expensive than EEG alone	Emerging research phase	(Cicalese et al., 2020)
Biological Fluids	CSF	0.94	90%	High Sensitivity and specificity; can detect AD progression	Invasive procedure, irreproducible diagnosis	Clinically validated, widely used	(Palmqvist et al., 2015)
	Plasma	0.89	83%	Less invasive; easy to collect	Lower Sensitivity and specificity, less correlation to AD	Emerging research phase	(Jiao et al., 2021)

Despite extensive research into new diagnostic methodologies and significant investment in the development of AD therapies, no treatments currently exist that effectively modify the disease course. There are two types of treatments for AD: symptomatic treatments and disease-modifying treatments. Symptomatic therapies improve the quality of life and cognitive function by addressing the effects of the disease while disease-modifying treatments aim to change the underlying biology of AD (Yiannopoulou & Papageorgiou, 2020). Current FDA-approved drugs fall under symptomatic treatments, including cholinesterase inhibitors (ChEIs) and anti- N-methyl-D-aspartate (NMDA) receptor drugs (Long & Holtzman, 2019). Acetylcholine (ACh) is an essential neurotransmitter in cognition and muscle activation. In AD, the loss of cholinergic neurons reduces ACh levels, leading to memory and attention problems. So, the use of ChEIs (like donepezil, rivastigmine, and galantamine) can prevent the breakdown of ACh, increasing its concentration in the brain (Lin et al., 2018; Long & Holtzman, 2019). On the other hand, NMDA receptors, which regulate calcium and sodium flow, are overactivated in AD due to high Aβ levels, causing excitotoxicity and neuron damage. Memantine, an NMDA receptor antagonist, blocks excessive activity, protecting neurons from further damage (Y. Zhang et al., 2016; Wang & Reddy, 2017).

Currently, over 100 compounds are evaluated in various clinical trial stages, with 20 completing stage 3; however, none have proven effective in slowing cognitive decline or enhancing overall functionality (Cummings et al., 2021). Many AD treatments have failed due to challenges in addressing advanced-stage patients where neuronal damage is already extensive and irreversible (R. Wang & Reddy, 2017; Y. Zhang et al., 2016). Consequently, new therapeutic approaches are aimed at earlier intervention, especially in preclinical stages, to maximize the potential for altering disease progression (Yiannopoulou & Papageorgiou, 2020). However, uncertainties in AD's pathophysiology and the variability due to genetic and lifestyle factors complicate treatment development. These limitations underscore the pressing need for further investment in innovative diagnostic methods, supporting early detection and enhancing therapeutic precision to improve patient outcomes (Mehta & Schneider, 2021).

1.3. Gut microbiota: a challenge as a biomarker for Alzheimer's Disease

The ongoing search for new biomarkers for detecting and treating AD has now focused on the role of gut microbiota. Over the past two decades, gut microbiota has garnered significant scientific interest as a potential factor in preventing and managing various disorders (Park & Kim, 2023). Nowadays, the gut microbiota is recognized for its critical role in influencing brain health. Its diverse composition and its alteration (gut dysbiosis) have been linked to neurodegenerative diseases such as AD (Bostancikliоğlu, 2019; Pistollato et al., 2016). Several risk factors can compromise the integrity of the gut microbiota (Figure 1.1), including aging, poor diet, sedentary lifestyles, impaired sleep quality, genetics, and others (Thu Thuy Nguyen & Endres, 2022).

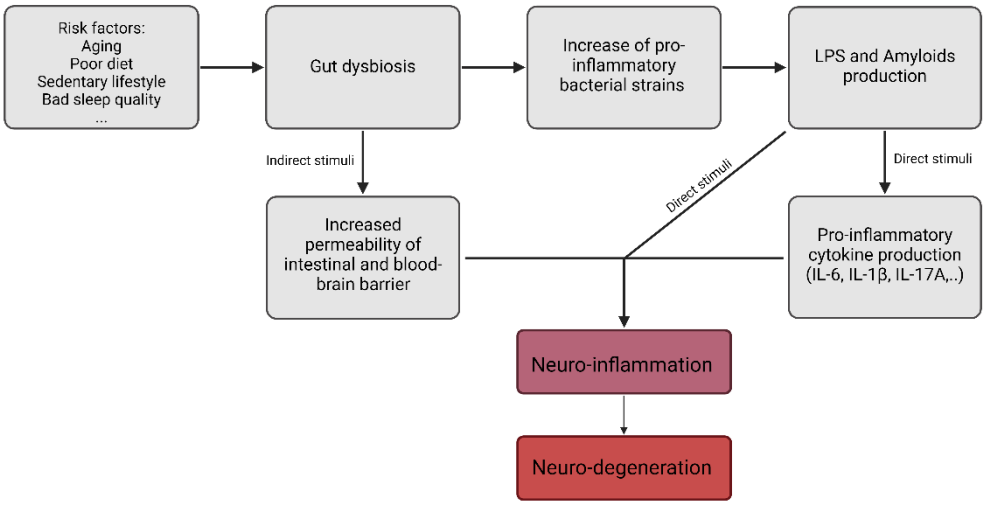

Figure 1.1 Mechanisms from which gut microbiota can lead to neurodegeneration adapted from Philip Mani et al., 2024.

Research on gut microbiota has consistently shown that it changes with age, with elderly individuals experiencing a decline in beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* compared to younger populations (Kossowska et al., 2024). These species produce γ-Aminobutyric acid (GABA), a key inhibitory neurotransmitter that supports cognitive function(Yunes et al., 2016). Reduced GABA production has been linked to synaptogenesis disorders, depression, and cognitive decline (Strandwitz, 2018).

Diet significantly influences the gut microbiota. Specifically, the Western diet, high in saturated fats and added sugars, is an established risk factor for AD (Więckowska-Gacek et al.,

2021). Several studies have indicated that high-fat diets reduce beneficial species while increasing harmful ones, promoting cognitive decline and dementia via oxidative stress, neuronal apoptosis, and increased Aβ deposition in the brain (Nam et al., 2017; Dodge et al., 2012; Więckowska-Gacek et al., 2021).

Physical exercise is an effective strategy for preventing dementia and neurodegenerative diseases, as it has been shown to provide neuroprotective effects through the reduction of pro-inflammatory species, such as *Clostridium* and *Eubacterium,* and decreasing Aβ deposition in the brain (Abraham et al., 2019; Wheeler et al., 2017). Furthermore, poor sleep quality is also a crucial risk factor for neurodegenerative diseases (Abraham et al., 2019). Studies in mice have shown that anesthetic sleep increases Aβ clearance (Xie et al., 2013), while sleep deprivation reverses this effect (Ooms et al., 2014). Partial sleep deprivation also affects the Firmicutes: Bacteroidetes ratio, increasing the abundance of families such as *Coriobacteriaceae* and *Erysipelotrichaceae* and lowering the abundance of *Tenericutes* (Benedict et al., 2016).

These changes in the gut microbiota, known as gut dysbiosis, lead to a decrease in beneficial anaerobes such as *Bifidobacterium* and *Lactobacillus* and cause chemical and immunological changes in the intestinal region (Nagpal et al., 2018). Such abnormalities impair the intestinal and brain-blood barriers, increasing their permeability and allowing pathogenic microorganisms and harmful metabolites to enter the bloodstream (Hashim & Makpol, 2022). Consequentially, these toxins reach the brain and create harmful reactions (Sharon et al., 2016).

On the other hand, dysbiosis also increases the presence of pro-inflammatory bacterial strains, producing harmful metabolites such as lipopolysaccharides (LPS) and amyloids. These trigger both direct and indirect inflammatory responses, increasing permeability in the intestinal and blood-brain barriers, ultimately leading to neuroinflammation, reduced neural plasticity, neurodegeneration, and nerve cell death (Shen et al., 2020; Askarova et al., 2020). The literature reported that AD patients possess higher levels of LPS in blood and brain tissues than individuals without mental impairment (R. Zhang et al., 2009; Zhao et al., 2017). Furthermore, intestinal microorganisms also produce and excrete functional amyloid peptides that are beneficial to the bacterial cell, with some species, such as *Escherichia coli* and *Bacillus subtilis*, secreting substantially more significant amounts of the peptide (Hufnagel et al., 2013). These bacterial metabolites are like the human AB42 peptide, allowing them to interact with the human toll-like receptor 2 (Lin et al., 2018). This interaction, in turn, activates the release of pro-inflammatory cytokines like IL-1β, IL-6, and TNF-α, as well as the pro-inflammatory factor

NFκB, which is known to trigger neurodegeneration in the brain of an AD patient (Köhler et al., 2016; Rapsinski et al., 2015).

Evidence suggests that gut microbiota can influence brain functions, and significant differences have been found between the taxonomic composition of healthy controls and AD patients (Table 1.2).

Table 1.2 Bacteria altered in MCI or AD patients compared to controls in several studies. HC – healthy control; MCI – mild cognitive impairment patients; Aβ+ - brain amyloidosis positive; Aβ- - brain amyloidosis negative.

Sample size	Sample characteristics			Location	Sequencing method	Bacteria altered	Ref
	Group	Age	Sex (M/F)				
N = 83	40 MCI (Aβ+) 33 MCI (Aβ-) 10 HC	71 70 10	20/20 16/18 4/6	Eastern Lombardy, Italy	*qPCR*	↑*Escherichia/ Shigella* (genus) ↓*Eubacterium* (genus)	(Cattaneo et al., 2017)
N = 50	25 Dementia 25 HC	69 71	7/18 8/17	Wisconsin, USA	16S rRNA	↑ Bacteroidetes (phylum) ↓ Firmicutes (phylum) ↓ *Bifidobacterium* (genus)	(Vogt et al., 2017)
N = 86	43 AD 43 HC	70 70	20/23 20/23	Chongqing, China	16S rRNA	↑ Actinobacteria (phylum) ↓ *Bacilli* (class) ↓ *Negativicutes* (class) ↓ *Bacteroidia* (class)	(Zhuang et al., 2018)
N = 97	33 AD 32 MCI 32 HC	75 71 77	19/14 14/18 16/16	Hangzhou, China	16S rRNA	↑ Proteobacteria (phylum) ↑ Enterobacteriaceae (phylum) ↑ *Gammaproteobacteria* (order) ↑ *Enterobacteriales* (family) ↓ Firmicutes (phylum)	(Liu et al., 2019)
N = 42	20 AD 12 MCI 20 HC	73 71 69	9/11 6/6 8/12	Bangkok, Thailand	16S rRNA	↑ *Enterobacteriaceae* (family) ↑ *Clostridiaceae* (family) ↑ *Bacteroides* (genus)	(Wanapaisan et al., 2022)

N = 17	11 MCI 6 HC	65	-	North Carolina, USA	16S rRNA	↑ Proteobacteria (phylum)	(Nagpal et al., 2019)
N = 96	31 MCI 65 HC	74 74	16/15 31/34	Taipei, Taiwan	16S rRNA	↑ *Phoeca* (genus) ↑ *Gemella* (genus) ↑ *Anaeroglobus* (genus) ↑ *Cloacibacillus* (genus) ↑ *Lactococcus* (genus) ↑ *Flavonifractor* (genus) ↑ *Cetobacterium* (genus) ↑ *Eubacterium* (genus) ↑ *Lactiplantibacillus* (genus) ↓ *Ruminococcus* (genus) ↓*Butyricimonas* (genus) ↓*Oxalobacter* (genus)	(Fan et al., 2023)

Over the last decade, several research has been conducted on the intestinal microbiome and AD, as seen in Table 1.2. Notably, some findings include an increase in pro-inflammatory bacteria such as *Escherichia-Shigella* and a decrease in beneficial bacteria including *Bifidobacterium* and *Butyricimonas* (Cattaneo et al., 2017; Vogt et al., 2017). Other studies have reported increased levels of Proteobacteria and Bacteroidetes, while the Firmicutes populations decreased (Zhuang et al., 2018; Liu et al., 2019).

In this way, these studies enable the creation of a database of the bacterial species present in patients without and with pathology (and at different stages of the disease). Researchers can then use this data to develop disease risk prediction models (Zheng et al., 2020), chronic disease surveillance (L. Zhao, 2013) or comparing disease prevalence (You et al., 2023). While statistical analysis may allow us to draw some conclusions, machine learning algorithms may uncover unseen patterns in such large and complex datasets. This rich data, combined with the application of ML algorithms, holds immense potential to reveal patterns that conventional methods might overlook. Similarly, microbiome research has begun leveraging similar techniques, highlighting the necessity of precise microbial data acquisition. By integrating insights from large-scale datasets with advanced sequencing methods such as 16S rRNA sequencing and shotgun metagenomics, researchers can further unravel the intricate relationships between microbial communities and disease progression (Ranjan et al., 2016).

16S RNA sequencing is a targeted approach that amplifies and sequences the 16S ribosomal RNA gene, which contains nine hypervariable regions demonstrating considerable and differential sequence diversity among bacteria (Waechter et al., 2023). This approach is more accessible for large-scale studies since it is more cost-effective. However, its simplicity comes at the cost of taxonomic resolution. It only allows for identifying bacteria at the genus level and does not identify functional genes. Moreover, it is prone to primer bias, where some bacteria taxa may need to be more represented (Paul, 2023).

On the other hand, shotgun metagenomic, or whole genome shotgun, sequences all the genetic material present in a sample, including bacterial, archaeal, viral, fungal, and host DNA (Quince et al., 2017). It is a more comprehensive method, as it can identify the strain level and functional information, such as genes associated with metabolic pathways, antibiotic resistance, and other microbial traits (Quince et al., 2017). However, it is a more extensive and costly process, and relative abundances inferred vary significantly depending on the DNA extraction and sequencing protocol used (Lang et al., 2013).

Overall, microbial profiles and functional attributes detected through 16S rRNA sequencing and shotgun metagenomics hold significant potential to serve as diagnostic biomarkers, offering insights into health and disease states. These microbiota-derived biomarkers could enhance personalized diagnostics and therapeutic monitoring approaches as research progresses.

1.5. Machine learning as a diagnostic tool

ML has become a transformative tool for microbiome research in recent years, primarily due to the vast and complex data it generates, which often reveals patterns undetectable by traditional analysis (Elmassry et al., 2024). ML algorithms excel at identifying these complex patterns by building and optimizing predictive models based on input data, making them ideal for analysing microbiome composition, functional potential, and even associations with disease states (Pistollato et al., 2016).

ML methods can be categorized primarily into supervised and unsupervised learning approaches, as pictured in Figure 1.2.

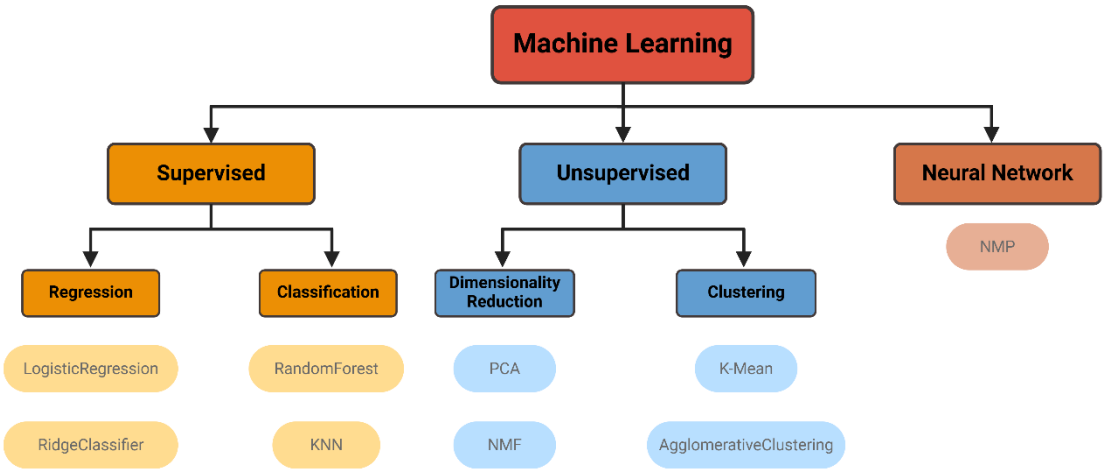

Figure 1.2 Overview of Machine Learning techniques.

Supervised learning techniques rely on labelled data, providing algorithms with known input-output relationships to help predict outcomes based on new data. Examples include classification models used for disease prediction, such as support vector machines (SVMs), random forests, and neural networks. In contrast, unsupervised learning models operate on unlabelled data, detecting inherent patterns or clusters within the dataset (Mathieu et al., 2022;

Papoutsoglou et al., 2023). This is especially useful in microbiome studies, where specific microbial patterns might emerge in disease versus healthy states, as seen in clustering analyses and principal components analysis (PCA) (X. Chen et al., 2022; Elmassry et al., 2024).

1.6. Supervised learning

In many cases where there is a relationship between a taxonomic abundance and an increased potential of certain diseases, it is possible to use that microbiome data to assess the possibility of that disease. Supervised learning methods can be further divided into two groups according to the problem: regression and classification problems. In these models, labelled datasets are used, meaning every entry to train the model has a known outcome.

Classification methods assign input data to predefined categories, such as "AD patient" or "Healthy Control." For instance, one commonly used classification method is RandomForestClassifier (RF) (Figure 1.3). It is an ensemble learning method that builds multiple decision trees using different data subsets and merges them to produce a more accurate and stable prediction. Each tree starts on a node, which is the feature that best separates the data between classes and proceeds to keep splitting itself into nodes using different features until reaching a final decision. A study by Pasolli et al. used ML models on metagenomics data to detect certain diseases and achieved accuracy scores over 0.8 using RF when applied to cirrhosis, colorectal, and bowel disease (Pasolli et al., 2016). This method provides a robust classification framework and offers insights into which microbial features are most important in distinguishing between disease states.

Regression techniques, on the other hand, are used to predict continuous outcomes based on input features; some models, like LogisticRegression (LogReg), calculate the probability of the input belonging to a particular class (values from 0 to 1) (Figure 1.3). LogReg is a straightforward method that models the relationship between variables and a predicted outcome by fitting an equation to the observed data. RidgeClassifier is an extension of LogReg that introduces a regularization term to the data to prevent it from becoming too complex and, thus, from overfitting (Su et al., 2012). An overfitted model is adapted to the training data and performs poorly on new, unseen data. It has lost its generalization ability (Hawkins, 2004).

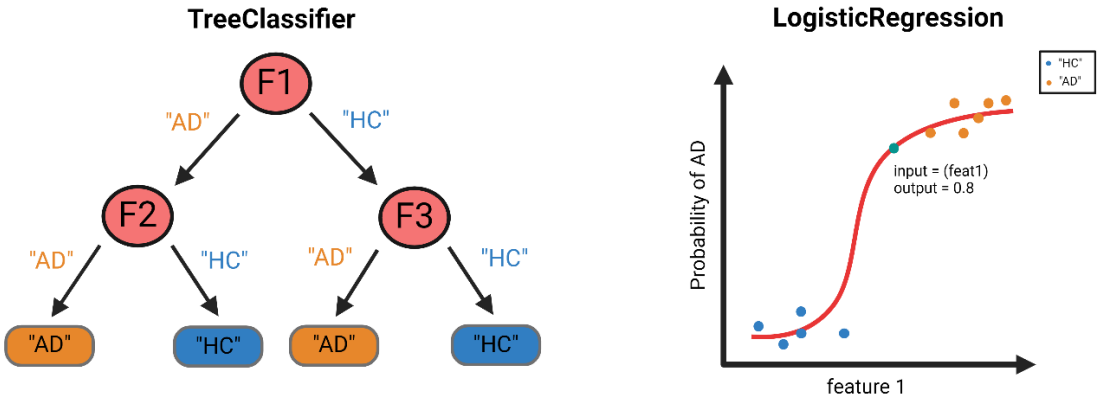

Figure 1.3 (Left) Illustration of a classification tree, each feature (F1, F2, F3) is represented by a circle, and decision outcomes ("AD" and "HC") are shown in the rectangles. (Right) A LogReg graph fitted equation is illustrated in red, and the input variable is shown in green.

1.7. Unsupervised learning and preprocessing

Unlike supervised ones, unsupervised learning methods do not rely on labelled data and are essential to discovering underlying patterns and structures within a dataset. However, since these models solely group data based on similarities and not based on their "true" category, they are not used to classify but rather to perform preprocessing on the data.

Since some datasets are too high-dimensional and complex even for machines, some steps can be taken to reduce their size to less and more valuable components that the model later analyses. Preprocessing may include cleaning the data, selecting the most relevant features, or transforming the data. Unsupervised learning methods fall in the latter category as they can transform the data into distinct groups (clustering) or transform the high-dimensional data into lower-dimensional by identifying new axes (dimensionality reduction) (Figure 1.3).

Clustering techniques group data points based on their similarities, enabling the identification of natural groupings within the data. For example, K-Means clustering is a method that partitions data into a K number of clusters, with each data point assigned to the nearest cluster centre or centroid (Figure 1.4). To optimize itself, the algorithm iteratively adjusts the centroids to minimize the sum of squared distances between data points and their respective centroids.

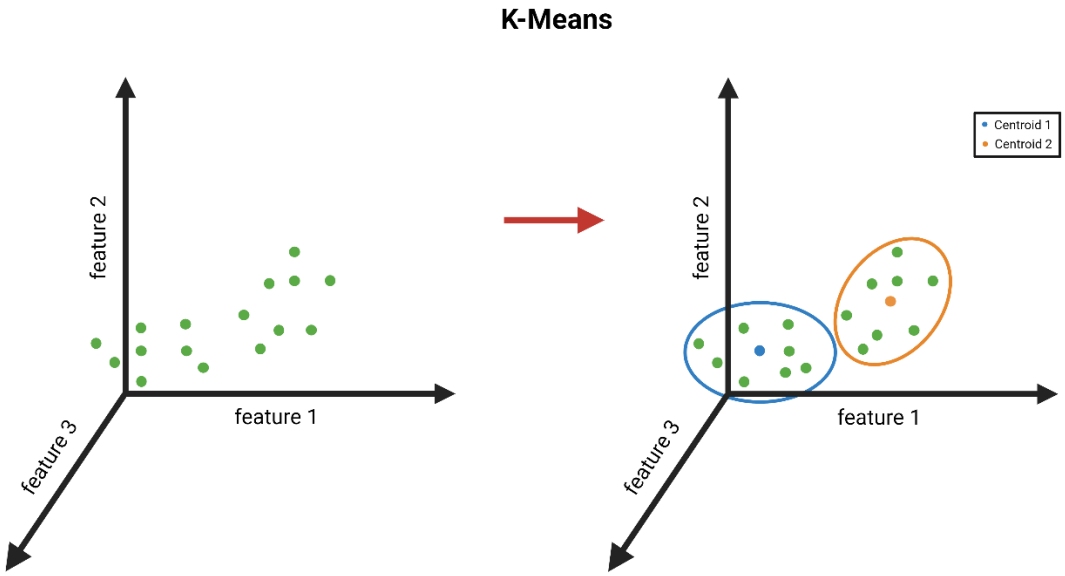

Figure 1.4 Illustration of K-Means with 2 clusters. Green circles represent data points; blue and orange circles represent centroids.

Dimensionality Reduction techniques are used to simplify high-dimensional datasets by transforming them into a smaller set of variables that still capture most of the original information. PCA does this by creating a new, uncorrelated set of features that explain most of the variance of the data. This method is also beneficial for visualizing and analysing complex, high-dimensional microbiome data (Hotelling, 1933; Zhang et al., 2013).

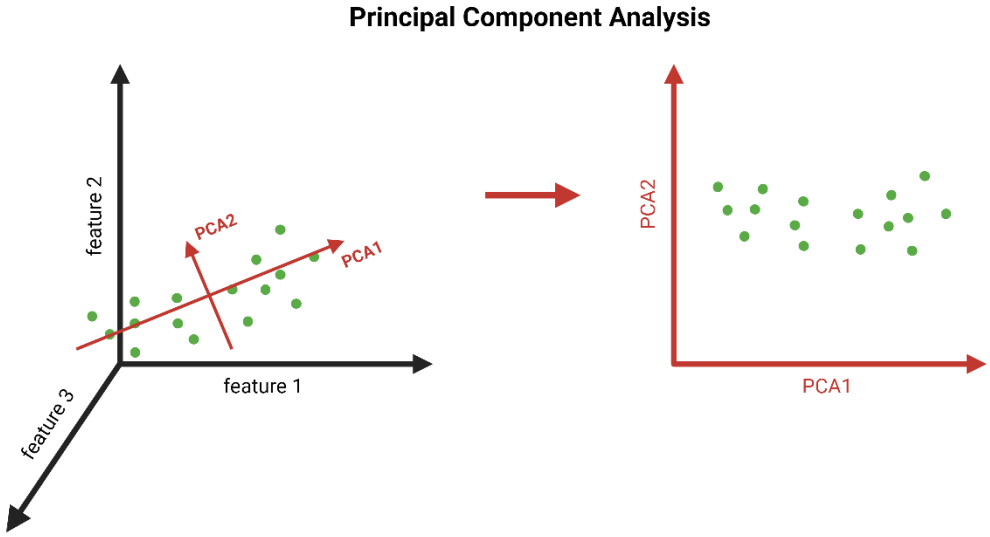

Figure 1.5 Principal Component Analysis with two principal components illustration. Green circles represent data points, and red arrows represent principal components.

1.8. Neural Networks

Neural networks, such as the Multi-layer Perceptron (MLP), are powerful ML models composed of a network of interconnected layers of neurons, like the structure of the human brain (Balcazar et al., 1997). Each neuron applies a mathematical transformation, using internal weights, to the input. A process called backpropagation adjusts the (Wu & Feng, 2018) to maximize accuracy (Wu & Feng, 2018). These versatile learning models can be trained on labelled and unlabelled data, allowing them to be utilized in regression and classification problems. Figure 1.6 Demonstrates a neural network consisting of an input layer, which receives the data, two hidden layers where the information is processed, and an output layer where the output of the result is realised (Tang et al., 2016).

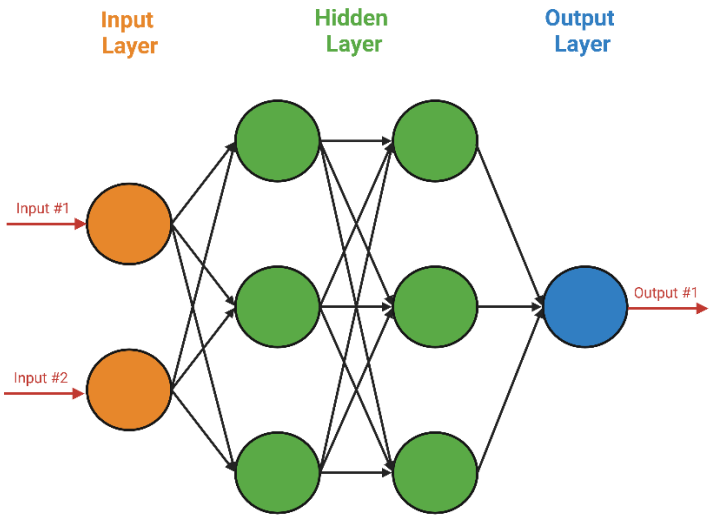

Figure 1.6 Illustration of a neural network with two hidden layers.

More recently, the application of ML algorithms to gut microbiota databases, often combined with other data types such as metagenomics or biomarkers, has opened new avenues to decipher the complex correlations between these variables and AD. As highlighted in Table 1.3, these emerging studies promise to improve our understanding and provide a diagnostic tool. However, while some studies have reported encouraging results, there remains significant room for improvement to realize the full potential of these methods in clinical settings.

Table 1.3 Studies that apply ML algorithms to gut microbiota data for AD classification.

Study	Algorithms compared	Number of subjects	Cross-validation method	Prediction performance	Ref.
Gut microbiota data for AD prediction	XGBoost	206	10-fold cross-validation	AUC = 0.84	(D'Aquila et al., 2021)
Gut microbiota data for AD prediction	RF, XGBoost	256	10-fold cross-validation	AUC = 0.72 Accuracy = 0.80	(Ma et al., 2021)
Gut microbiota + biomarker data for AD prediction	RF, LogReg	180	10-fold cross-validation	AUC = 0.68 Accuracy = 0.75	(Marizzoni et al., 2023)
Gut microbiota + functional data for AD detection	Ensemble Learning (LogReg)	175	10-fold cross-validation	AUC = 0.80	(Laske et al., 2022)

1.9. Goals and workflow

This thesis sets out to delve into the potential of the intestinal microbiome as a discriminatory factor for AD. First, a statistical analysis is performed, followed by an extensive exploration of various ML models to evaluate their effectiveness as diagnostic instruments. The ultimate objective is to identify microbial markers linked to AD and create a diagnostic tool that could simplify and accelerate the diagnostic process compared to conventional methods.

A simple statistical analysis was conducted on the dataset to gain an initial understanding of the taxonomic profiles, as illustrated in Figure 2.1 The data underwent preprocessing, using various techniques to prepare it for ML models. Multiple classifiers were then applied to the preprocessed data to identify the most effective model for distinguishing between AD patients and controls.

This model has the potential to fast-track the diagnostic process, offering a non-invasive, cost-effective alternative to traditional diagnostic methods, such as neuroimaging and cerebrospinal fluid analysis. By relying on microbiome profiles, the model could simplify diagnosis, requiring fewer complex or invasive tests, and could be easily integrated into clinical settings, enabling earlier detection and potentially improving patient outcomes. This would significantly advance over more invasive and costly procedures currently in use.

2. Materials and Methods

Figure 2.1 illustrates the methodology phases for analyzing and classifying AD based on gut microbiota data. Data from 175 subjects (100 healthy controls, 75 AD patients) was collected, statistically analyzed, and preprocessed before ML classification. Classification performance was assessed using Train-Test Split, 10-fold Cross-Validation, and Leave-One-Out Cross-Validation (LOOCV) to ensure robust model evaluation. Finally, the best-performing LOOCV model was selected for feature discriminative power analysis, identifying features with the highest individual capacity to distinguish between AD and healthy control (HC) groups.

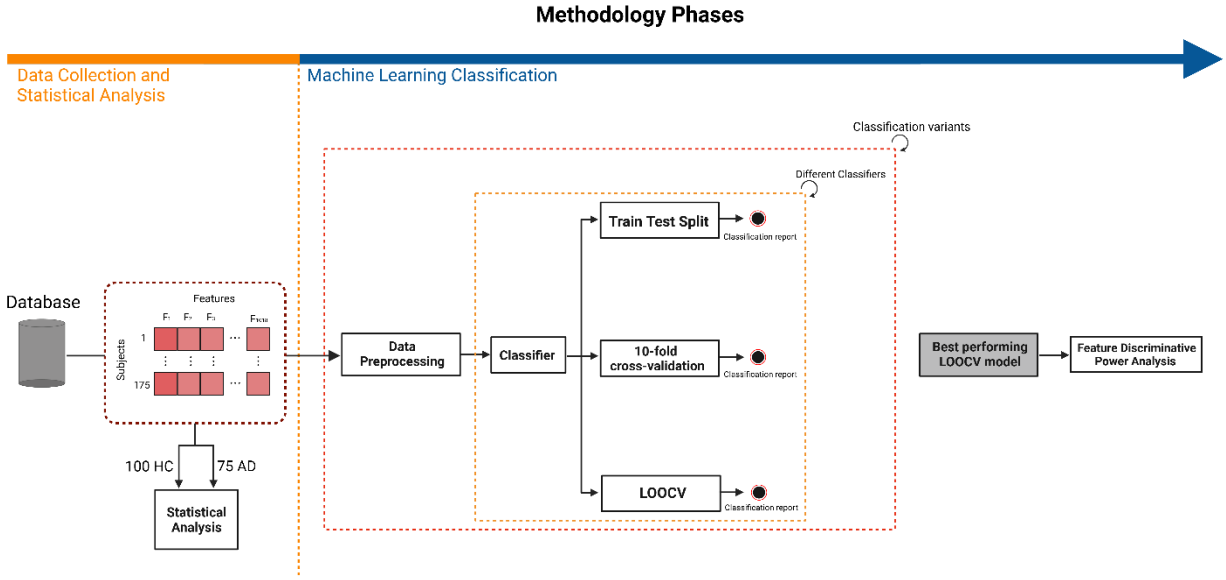

Figure 2.1 Methodology phases and workflow diagram.

2.1. Experimental setup

For this work, Python (version 3.12.4) was used to perform statistical analysis and design, perform, and get discriminant reports from a set of ML models.

2.2. Dataset

The dataset adopted in this study originates from the publication "Signature of Alzheimer's Disease in Intestinal Microbiome: Results From the AlzBiom Study" by Laske *et al.*, which is part of the AlzBiom study, an observational longitudinal research initiative

conducted at the Section for Dementia Research within the Department of Psychiatry and Psychotherapy in Tübingen (Laske et al., 2022). This dataset includes demographic information (age and sex), clinical data (BMI and presence of APOE4 alleles), and intestinal microbiome data from 175 individuals. This sample pool is comprised of 100 cognitively HC and 75 amyloid-positive AD participants who also met the NIA-AA core clinical criteria for probable AD dementia.

To generate the intestinal microbiome dataset, stool samples were collected and immediately processed for DNA extraction. Subsequently, shotgun metagenomics sequencing was performed, and the reads were processed, followed by metagenomics assembly and taxonomic classification of microbial communities. The results were normalized to represent the relative abundance of taxonomic units in hits per million reads (HPM).

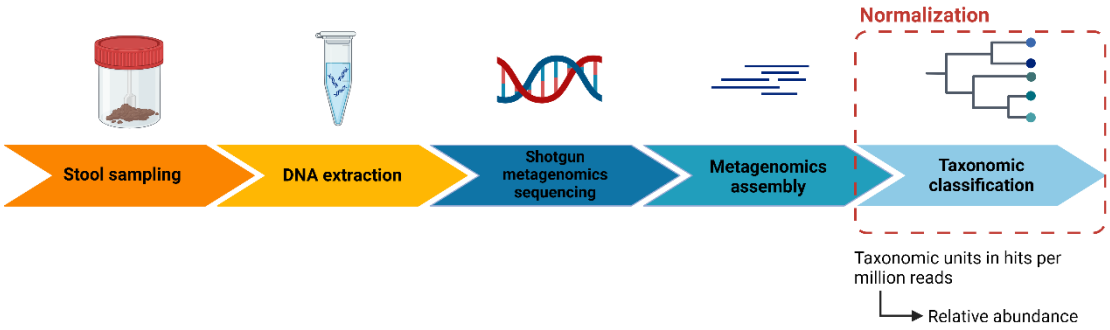

Figure 2.2 Workflow for forming the metagenomics database in "Signature of Alzheimer's Disease in Intestinal Microbiome: Results From the AlzBiom Study." by Laske *et al.*, 2019.

2.3. Statistical Analysis

A statistical analysis is valuable for verifying foundational patterns and distributions in the data, ensuring robust statistical assumptions for the subsequent ML analysis. This approach establishes a clear structure for extracting targeted insights, facilitates meaningful comparisons, and highlights trends critical to interpreting microbiome-related variations. The statistical analysis tests were performed using Sklearn's and Scipy's libraries (Pedregosa, 2011). A confidence level of 95% was applied and the homogeneity of variances were assessed using Levene's test.

2.3.1. Clinical Demographics

To begin understanding the demographic characteristics of the study participants, the clinical variables "Age," "Sex," and "APOE4" were analysed as categorical factors, while "BMI" was considered a continuous variable. The chi-squared test was applied to the categorical variables to assess group differences, while the Student's t-test was used for the constant variable.

2.3.2. Alpha Diversities and Gut Microbiota Composition

Alpha diversity analysis is crucial in understanding the gut microbiota as it helps assess the overall microbial richness and balance within a sample. By quantifying the diversities, we can identify potential variations in microbial composition that may link gut dysbiosis with AD.

To assess alpha diversity, both the Shannon and Simpson indices were applied with the following formulas:

$$H' = -\sum_{i=1}^{S}(p_i \times (\ln p_i)) \quad (1)$$

$$D = 1 - \sum_{i=1}^{S}(p_i^2) \quad (2)$$

The Shannon Index measures the uncertainty in predicting the species of an individual randomly selected from a dataset, considering both species richness and evenness. In formula (1), H' represents the Shannon Index, S is the total number of species, and pi is the proportion of individuals of species *i*. Higher values of the Shannon Index indicate greater diversity.

The Simpson Index measures the probability that two individuals randomly selected from a sample belong to the same species. It is more sensitive to evenness than species richness. In formula (2), *D* represents the Simpson Index, S is the total number of species, and p_i is the proportion of individuals of species *i*. The Simpson Index is inverted to 1 − D to make higher values indicate greater diversity. Significant differences in diversity between groups were evaluated using Student's t-test.

The last statistical test was performed on the taxonomic data to identify significant differences between groups' microbial profiles. First, the Kolmogorov-Smirnov test was applied, followed by either the Student's t-test (for normally distributed data) or the Mann-Whitney U test (for non-normally distributed data) to compare differences in taxonomic features between groups.

2.4. Prediction Approach via Machine Learning Algorithms

Before proceeding with the rest of the analysis, the numerical features "BMI" and "Age," along with the taxonomic data, were normalized using the MinMaxScaler from the sklearn library (Pedregosa et al., 2011). This process transformed the distribution of each feature to a range between 0 and 1. Unlike StandardScaler, which standardizes features based on their mean and standard deviation, MinMaxScaler preserves the relationships between data points and accommodates features with non-normal distributions, making it a more suitable choice for uniform scaling without altering data interpretation (Ahsan et al., 2021).

2.4.1. Feature selection and dimensionality reduction techniques

A series of feature selection and dimensionality reduction techniques were applied to the dataset to optimize the performance of the ML models and enhance interpretability. The goal was to identify and retain the most relevant features for class discrimination while minimizing noise and redundancy.

Several feature selection methods were applied to reduce the dimensionality of the dataset by removing irrelevant or redundant features. These techniques helped retain the features with the highest potential for class discrimination. The feature selection methods employed are shown in Table 2.1.

- SelectKBest (SKB): This method selected the top k features based on statistical tests (ANOVA F-test).
- VarianceThreshold (VT): This approach eliminated features with low variance, assuming features with slight variation do not contribute much to classification.
- SelectFromModel: Models such as RidgeClassifier, LogisticRegression, and RandomForestClassifier were used to rank features based on their importance.

- Recurse Feature Elimination (RFE): This method recursively removed the least essential features using Logistic Regression as the base estimator.

Table 2.1. Feature selector models and specific hyper-parameters were applied before classification.

Model	**Specific hyper-parameters**	
SelectKBest (SKB)	- k = [1, 2, 3, 4, 5, 10, 20, 40, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900] - function = mutual_info_classif	Selects subsets of k features with the best scores.
VarianceThreshold (VT)	- threshold = [0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5]	Selects subsets of features with variance above the threshold.
SelectFromModel – RidgeClassifier	- alpha = 0.1 - threshold = "median" - max_features = [1, 2, 3, 5, 10, 20, 50, 70, 100, 200, 300, 400]	Select features with importance above the median feature importance
SelectFromModel – LogisticRegression*	- penalty = "elastic net" - solver = "saga" - C = 0.1 - max_iter = 1000 - threshold = "median" - max_features = [1, 2, 3, 5, 10, 20, 50, 70, 100, 200, 300, 400]	
SelectFromModel – RandomForestClassifier	- n_estimators = 200 - max_depth = 15 - threshold = "median"	

RecursiveFeatureElimination – LogisticRegression	- n_to_select = [1, 2, 3, 5, 10, 20, 50, 70, 100, 200, 300, 400] - scoring = "roc_auc" - step = 0.1	Recursively selects subsets of n features.

The dimensionality reduction methods applied to simplify the dataset by transforming it into a lower-dimensional space while retaining the most informative aspects are shown in Table 2.2.

- Principal Component Analysis (PCA): PCA was used to reduce the dataset into orthogonal components that captured the majority of variance.
- Non-Negative Matrix Factorization (NMF): This technique decomposed the dataset into a set of non-negative factors.
- Isomap: This technique captured the manifold structure of the data by mapping it into a lower-dimensional space.

Table 2.2 Dimensionality reduction techniques and specific hyperparameters were applied before classification.

Dimensionality Reduction Techniques	Hyperparameters
Principal Component Analysis	- n_components = [0.7, 0.8, 0.9] - whiten = True
Non-Negative Matrix Factorization	- n_components = 10,20 50,100,200 - l1_ratio = 0.5 - alpha = 0.1 - max_iter = 1500 - init = nndsvd
Isomap	- n_components = [5,10,20,50,100,200] - n_neighbors = 5

2.4.2. Classification

After preprocessing, the datasets obtained from each feature selector were fed into eighteen machine-learning models, as shown in Table 2.3. This diverse set of supervised models was chosen to ensure a comprehensive classification performance evaluation, leveraging the strengths of various algorithms.

Table 2.3 Classifiers and specific hyperparameters are used for the prediction models.

Classifier	Hyperparameters
AdaBoostClassifier (AdaBoost)	n_estimators = 50 learning_rate = 1.0 algorithm = “SAMME”
BaggingClassifier (BaggC)	n_estimators = 10
DecisionTreeClassifier (DeTreeC)	max_depth = 5

ExTreeC (ExtraTreesClassifier)	n_estimators = 200
GaussianNB (GauNB)	Default
GaussianProcessClassifier (GauPro)	1.0 * RBF(1.0)
GradientBoostClassifier (GradBoost)	n_estimators = 200 learning_rate = 0.1 max_depth = 3
KNeighborsClassifier (KNN)	n_neighbors = 5
LinearDiscriminantAnalysis (LinDis)	Default
LinearSVC (LinSVC)	max_iter = 1000 C = 1.0
LogisticRegression (LogReg)	solver = 'lbfgs' max_iter = 1000
LogisticRegressionCV	cv = 5 max_iter = 1000
MLPClassifier (MLP)	alpha = 0.01 max_iter = 200
RandomForestClassifier (RF)	max_depth = 5 n_estimator = 100
SGDClassifier (SGD)	max_iter = 1000 tol = $1*e^{-3}$
SVC	kernel = 'rbf' C = 1 gamma = 'scale' probability = True
RidgeClassifier (Ridge)	alpha = 0.1 L1_ratio = 0.5
GradientBoostingClassifier	n_estimators = 100 learning_rate = 0.1 max_depth = 3

The ML models were evaluated using three different methods of validation:

- Train-test split**:** involved splitting the dataset into a training set containing 70% of the data, used to train the model, and a testing set containing the remaining 30% of the data, which was then used to obtain an evaluation report.
- 10-fold cross-validation: the dataset was split into ten equal parts (folds). The model was trained on nine folds, and the remaining fold was used for validation. This process

was repeated ten times, each producing an evaluation report, then averaged across all folds.

- LOOCV: used all but one sample as the training set, with the remaining sample used for testing. This process was repeated for each sample in the dataset, providing an exhaustive evaluation that ensures maximum usage of the available data and helps assess the model's performance on small datasets.

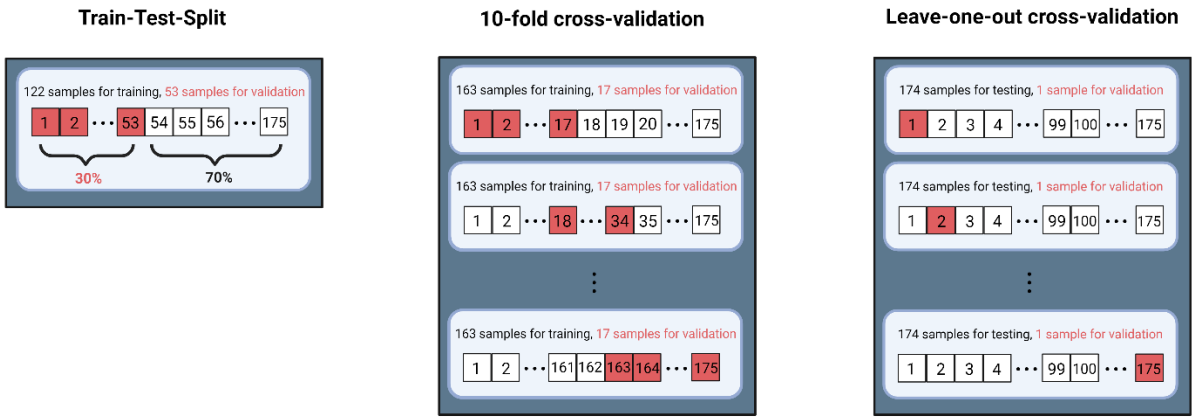

Figure 2.3 Comparison of model validation techniques: Train-Test Split, 10-Fold Cross-Validation, and LOOCV.

2.4.3. Classification metrics

Several metrics were calculated to evaluate the final model performance: *Accuracy*, AUC, *F1-Score*, *Precision*, *Recall* (Sensitivity), *Specificity,* and *G-Mean*.

Accuracy is defined as the ratio of correctly predicted labels to the total number of predicted labels, being described as:

$$Accuracy = \frac{TP + TN}{TP + TN + FP + FN} \quad (3)$$

where *TP* (True Positives) is the number of instances where the model correctly predicts AD cases, *TN* (True Negatives) is the number of instances where the model correctly predicts a healthy case, *FP* (False Positives) is the number of instances where the model incorrectly predicts an AD case, and *FN* (False Negatives) is the number of instances where the model incorrectly predicts a healthy case.

Precision and *Recall* are both based on true positives. *Precision* measures the proportion of correctly predicted AD cases to the total number of predicted AD cases (both correct and incorrect). *Recall*, also known as Sensitivity, measures the proportion of correctly predicted AD cases to the total number of actual AD cases in the dataset. Both equations are defined as:

$$Precision = \frac{TP}{TP + FP} \tag{4}$$

$$Recall = \frac{TP}{TP + FN} \tag{5}$$

*F*1-*Score* is the harmonic mean of *Precision* and *Recall*, providing a balanced measure that considers both false positives and false negatives. It is beneficial for imbalanced datasets.

$$F1\text{-}Score = 2 \times \frac{Precision \times Recall}{Precision + Recall} \tag{6}$$

Specificity is the ratio between correctly predicted healthy cases and every healthy case. It shows how many negative cases are being correctly identified by the model.

$$Specificity = \frac{TN}{TN + FN} \tag{7}$$

The geometric mean (*G-Mean*) provides a comprehensive measure of how well the model balances the identification of AD and healthy cases. A higher *G-Mean* indicates that the model performs well across both classes, ensuring predictive solid power for the disease and control groups.

$$G\text{-}Mean = \sqrt{Recall \times Specificity} \tag{8}$$

2.5. Feature discriminative power analysis

For this task, the individual discriminate power of the features used in the best-performing model, validated by LOOCV, was analysed, and a visual representation of the groups through PCA was generated.

The features selected in the preprocessing phase were individually evaluated using the classifier to quantify their ability to distinguish between groups based on the Area under the receiver operating characteristics curve (AUC) performance measurement. They were then ranked according to the values obtained, and the best receiver operating characteristic (ROC) curves were plotted. AUC values range from 0 to 1:

- AUC = 1: The feature perfectly distinguishes between control and AD groups.
- AUC = 0.5: The feature does not perform better than random chance.
- AUC <0.5: The feature performs worse than random guessing.

3. Results and Discussion

3.1. Statistical Analysis

3.1.1. Clinical and Demographic Analysis

Characteristics of the study sample are shown in Table 3.1. There was a statistically significant difference in the average age between the two groups, with the healthy controls being slightly older (mean age = 71.0 years) compared to the AD group (mean age = 68.84 years). Both sex and BMI remained equally distributed, with the latter constituting a BMI inside the range for a healthy older individual in both groups.

As for the ApoE4 allele, there was a significant difference between the two groups. In the HC group, the majority (68%) had no copies of the allele, while 28% had one copy and only 4% had two copies. In contrast, a much higher proportion of AD participants had at least one ApoE4 allele (43% with one copy and 7% with two copies). This is consistent with the meta-analysis by Farrer *et al.*, which demonstrated that 40-65% of Alzheimer's patients carry at least one copy of the ApoE4 allele, compared to 25-30% of the general population, with 2-3% carrying two copies (Farrer, 1997). These results underscore the solid genetic predisposition associated with the ApoE4 allele and AD.

Table 3.1 Clinical and demographics data of healthy controls and amyloid-positive AD participants.

	HC	**AD**	***p*-value**
N	100	75	
Age	71.0 ± 4.4	68.84 ± 8.6	0.0194
Sex (M/F)	54/46	34/41	0.326
BMI	25.76 ± 4.4	25.35 ± 4.3	0.530
ApoE4 (0/1/2)	68/28/4	25/43/7	<0.001

3.1.2. Alpha Diversities and Gut Microbiota Composition

The preliminary statistical analysis of Alpha diversity using the Shannon and Simpson indices shows no significant differences between the HC and AD groups. Normality tests

indicated that the Shannon index is usually distributed for both groups, whereas the Simpson index is not. The t-test for the Shannon index and the Mann-Whitney U test for the Simpson index returned *p*-values greater than 0.05, indicating no significant group differences in microbial diversity.

This quick statistical analysis suggests that basic diversity metrics do not distinguish AD participants from HC. Similar results have been observed in other studies. For example, Vogt et al. reported no significant difference between groups in Shannon or Simpson diversity indices. At the same time, Hung et al. reviewed several studies on AD and gut microbiota, noting that some reported decreased alpha diversity, especially in advanced stages of AD, while others showed no clear pattern (Jemimah et al., 2023; Vogt et al., 2017). Since microbial diversity alone does not differentiate the groups, the ML models will be crucial in identifying microbial markers or hidden relationships that might provide more insights into the gut microbiota's relationship with AD.

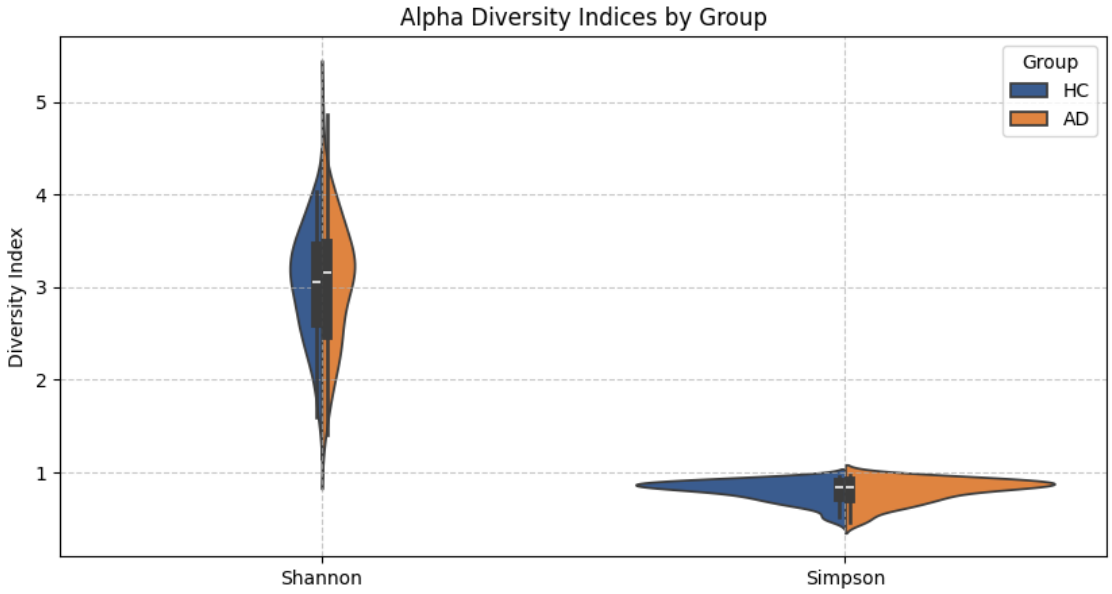

Figure 3.1 Shannon and Simpson's alpha diversity indices comparison between HC and AD groups.

The differential abundance analysis revealed that the AD participant's microbiome showed a significantly altered abundance of 5 taxa relative to the HC group, as shown in Figure 3.2.

The *Escherichia* and *Shigella* genera were the most differential between groups, appearing in overabundance in AD participants. Sometimes grouped as *Escherichia-Shigella* due to their genetic similarities, both genera are known for their pro-inflammatory properties,

being commonly associated with gut inflammation and intestinal dysbiosis, conditions These bacteria can breach the intestinal barrier, which can lead to systemic inflammation—exacerbating neuroinflammation via the gut-brain axis and contributing to the pathogenesis of AD. These findings are consistent with several other studies that reported an overrepresentation of *Escherichia-Shigella* in AD participants (Cattaneo et al., 2017; Zhuang et al., 2018).

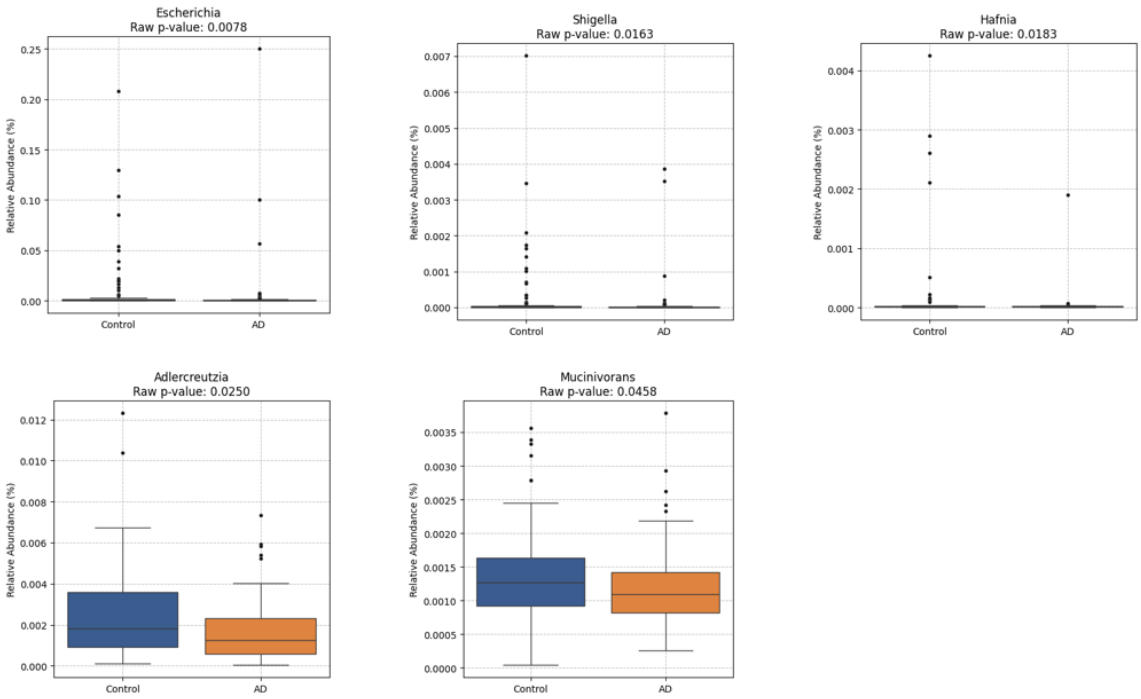

Figure 3.2 AD participants differentially represented bacterial genera compared to HC participants ($p < 0.05$).

The *Adlercreutzia* genus presented a significant decrease in AD participants. This bacterium has a role in metabolizing phytoestrogens, which are linked to anti-inflammatory and neuroprotective effects. Its reduction in AD participants suggests a reduced ability to manage inflammation. Researchers have also found a negative correlation between the ApoE4 allele and *Adlercreutzia*, as well as a loss of phytoestrogen-metabolizing bacteria such as this one among AD older adults with rapidly progressing dementia (Loew et al., 2023; Wojtuś et al., 2024).

Finally, the *Mucinivorans* and *Hafnia* genera also present a marginal abundance difference between the two groups. *Mucinivorans* is a bacterium breaking down mucin, a glycoprotein essential for maintaining the integrity of the gut's mucosal barrier. Some studies show that reducing these mucin-degrading bacteria in AD participants may suggest compromised gut barrier function (Li et al., 2024).

These findings provide compelling evidence that specific shifts in the gut microbiota, particularly involving bacteria associated with inflammation and mucosal integrity, are linked to AD. The overabundance of pro-inflammatory genera like *Escherichia-Shigella*, coupled with the reduced presence of beneficial mucin-degrading bacteria like *Mucinivorans*, supports the theory that gut dysbiosis may exacerbate systemic inflammation, potentially influencing neurodegenerative processes through the gut-brain axis.

3.2. Prediction Approach via Machine Learning Algorithms

In this study, ML algorithms were applied to assess the ability of microbiome and clinical data to differentiate AD patients from healthy controls (HC). The dataset was organized in a structured format, where each row represented an individual sample, and each column corresponded to a specific feature, including taxonomic profiles, APOE4 status, BMI, age, and sex.

Several feature selection methods were employed prior to model training to enhance the predictive power of the models. These methods were used to identify and retain the most informative features, reducing dimensionality and improving classification accuracy. The refined feature sets were then used in model development and evaluated through train-test split, 10-fold cross-validation, and LOOCV.

Overall, the ML models demonstrated positive predictive scores across the different validation approaches, with each method yielding distinct results that highlight variations in model stability and consistency. Tables 3.2, 3.3, and 3.4 present the five best-performing models for each validation approach: train-test split, 10-fold Cross-Validation, and LOOCV, respectively, ranking models by AUC score to measure their ability to discriminate between AD and HC.

The train-test split method yielded the highest-performing model with KNeighborsClassifier, using five selected features from the SelectKBest pre-processing method. This model achieved an AUC of 0.821, an accuracy of 0.792, and a precision-recall balance with a recall of 0.783 and an F1-score of 0.766. While the AUC is relatively strong, the accuracy reflects a moderate ability to distinguish between groups, suggesting that while the model performs well in general, individual classification may be variable.

Table 3.2 Performance metrics of the five best-performing ML models for AD and HC prediction using train-test split validation method.

Pre-processor	Classifier	Selected Features	AUC	Accuracy	Precision	Recall	F1-Score	G-mean
SelectKBest	BaggingClassifier	5	0.826	0.698	0.630	0.739	0.680	0.702
SelectKBest	KNeighborsClassifier	100	0.821	0.792	0.750	0.783	0.766	0.791
SelectKBest	KNeighborsClassifier	70	0.820	0.792	0.750	0.783	0.766	0.791
RFE_LogReg	BaggingClassifier	10	0.818	0.774	0.789	0.652	0.714	0.752
RFE_LogReg	DecisionTreeClassifier	5	0.817	0.679	0.650	0.565	0.605	0.658

Table 3.3 Performance metrics of the five best-performing machine ML models for AD and HC prediction using 10-fold Cross-Validation.

Pre-processor	Classifier	Selected Features	AUC	Accuracy	Precision	Recall	F1-Score	G-mean
SelectFromModel_RandomForest	AdaBoost	20	0.828	0.731	0.719	0.613	0.662	0.709
SelectFromModel_LassoCV	RandomForestClassifier	5	0.824	0.720	0.663	0.707	0.684	0.718
VarianceThreshold	RandomForestClassifier	0.01	0.824	0.754	0.722	0.693	0.707	0.745
SelectFromModel_LassoCV	RandomForestClassifier	10	0.823	0.726	0.680	0.680	0.680	0.719
SelectFromModel_LassoCV	RandomForestClassifier	20	0.820	0.749	0.696	0.733	0.714	0.747

Table 3.4 Performance metrics of the five best-performing ML models for AD and HC prediction using LOOCV.

Pre-processor	Classifier	Selected Features	AUC	Accuracy	Precision	Recall	F1-Score	G-mean
RFE_LogReg	AdaBoost	50	0.880	0.886	0.887	0.840	0.863	0.879
RFE_LogReg	RidgeClassifier	50	0.880	0.886	0.887	0.840	0.863	0.879
RFE_LogReg	LinearDiscriminantAnalysis	50	0.848	0.851	0.827	0.827	0.827	0.848
RFE_LogReg	LinearSVC	50	0.837	0.840	0.813	0.813	0.813	0.836
SelectFromModel_LogReg	MLPClassifier	50	0.833	0.840	0.831	0.787	0.808	0.832

In 10-fold Cross-Validation, our best model, RandomForest as pre-processor and AdaBoost as classifier trained with 20 selected features, achieved an AUC of 0.828, an accuracy of 0.731, and an F1-score of 0.662. These results show that the model maintains stable performance, balancing Sensitivity and accuracy.

The LOOCV method revealed the highest AUC scores, with the top models achieving AUCs of 0.880 using Recursive Feature Elimination with Logistic Regression for feature selection and AdaBoost classifier on 50 features. This model had comparable metrics, showing an accuracy of 0.886 and balanced recall and F1 scores, reflecting that LOOCV could identify strong feature combinations and classifiers with consistent individual predictive power. Additionally, a G-mean value of 0.879 indicates model robustness across both groups, capturing pattern differences associated with AD and HC.

Given the modest sample size of 175 participants, uncovering meaningful patterns and generalizable relationships within the data presents inherent challenges. Smaller datasets, especially in studies examining complex systems like the gut microbiota and its association with AD, tend to increase the risk of model overfitting and reduce the reliability of results, as they capture only a fraction of the natural variability present in larger populations (Domingues et al., 2018). In this case, LOOCV is particularly advantageous as it utilizes each sample in the testing phase, minimizing bias and allowing for an exhaustive evaluation of the model's generalization ability (Ramezan et al., 2021). Additionally, LOOCV produced the highest G-mean scores, indicating robust performance across AD and control groups, which is critical when handling imbalanced datasets (Douzas et al., 2018). By contrast, the train-test split approach, which divides data into distinct training and testing sets, proved less effective for our dataset. While computationally efficient, this method depends heavily on the specific split, leading to high variability in results and diminished accuracy, especially in smaller samples where each data point contributes significantly to the overall findings (Tan et al., 2021). In this study, the train-test split delivered lower scores across accuracy, precision, and G-mean metrics, underscoring its limitations in stability and consistency.

In comparing our best-performing model under 10-fold Cross-Validation with similar models from a referenced study, it's evident that our model demonstrates competitive results but with slightly lower discriminative power. The highest Area Under the Receiver Operating Characteristic Curve (AUROC) achieved with our dataset was 0.83, attained using the SelectFromModel_LassoCV pre-processor paired with the RandomForest classifier alongside a feature subset of 20 taxa. This outcome is close to but does not exceed, the referenced study's

top models, which reported AUROCs of 0.81, 0.83, and 0.92 with various combinations of functional data, including Gene Ontology features, KEGG Orthology features, and an ensemble model incorporating clinical data. These features provide richer context as they directly relate to microbial metabolic and regulatory pathways involved in AD pathophysiology (Xing et al., 2016). By incorporating functional data, models can capture potential mechanistic links between specific microbiome functions and neurodegeneration, offering a more nuanced understanding than taxonomic data alone.

All models built using dimensionality reduction techniques exhibited lower predictive scores than those using standard feature selection methods. The best-performing model among these was PCA combined with LinearSVC, which achieved an AUC of 0.67 and an accuracy of 0.65. This suggests that while dimensionality reduction helps to simplify the model by reducing feature complexity, it may also remove crucial discriminatory information, leading to decreased classification performance in distinguishing between AD and healthy controls in our dataset.

The model demonstrates promising predictive accuracy using microbiome taxonomic data alongside select clinical parameters, highlighting its potential as a streamlined diagnostic aid. While integrating functional data could provide additional insights into microbial metabolic pathways and gene functions that correlate with disease states, it would also add complexity to the analysis pipeline. Each sample would require more intensive sequencing and computational processing, thereby increasing the cost and time needed for diagnosis. Given that our secondary aim is to establish a cost-effective, non-invasive tool for early AD diagnosis, we focused on balancing diagnostic precision with practical feasibility. By relying primarily on taxonomic and clinical features, the current model remains aligned with this aim, allowing for faster implementation in clinical settings and potentially expediting the diagnostic timeline. A model that requires fewer resources yet achieves reliable performance would represent a valuable improvement over traditional, more invasive diagnostic approaches, facilitating earlier and more accessible patient care without the barriers of high-cost or resource-heavy procedures.

3.3. Feature individual discriminative power analysis

In this section, we examine the individual discriminative power of key features within our dataset, assessing their ability to distinguish AD patients from HC. Figure 3.3 presents ROC

curves for the top 10 features with the highest AUC scores, providing insights into their standalone predictive capability.

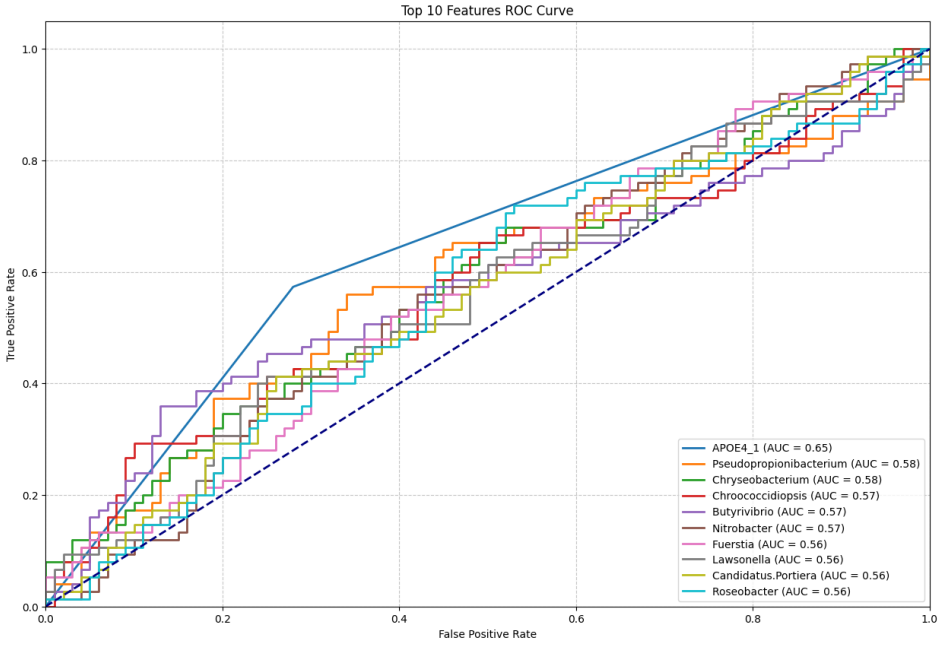

Figure 3.3 ROC curves for top 10 features for AD classification.

The feature APOE4_1 (presence of one ApoE4 allele) stands out with an AUC of 0.65, making it the most predictive feature of the dataset. However, this relatively low value makes its predictive power moderate rather than definitive. The remaining features, including bacterial taxa such as *Roseobacter*, *Chryseobacterium*, and *Butyrivibrio*, have low AUC values, ranging from 0.56 to 0.58. These are slightly above the baseline of 0.50, representing a random chance, indicating that these microbial features cannot distinguish between AD and HC groups.

The *Butyrivibrio* genus is known to produce byturate, a short-chain fatty acid with anti-inflammatory properties that helps to maintain the integrity of the blood-brain barrier and reduce neuroinflammation. Studies have shown that AD elders are characterized by a lower proportion of key butyrate-producing species, such as members of the *Butyrivibrio* and *Flavonifractor* genera (Fan et al., 2023; Haran et al., 2019).

4. Conclusion

This thesis work explores microbial markers for AD within the gut microbiota's taxonomic profile, applying ML methods to distinguish AD patients from healthy controls. Our sample of 175 participants, including amyloid-positive AD patients and healthy controls, revealed significant age and ApoE4 allele differences, with a higher prevalence of ApoE4 in the AD group, consistent with known AD risk factors. However, no significant differences were observed in sex, BMI, or microbiome alpha diversity between groups, aligning with prior findings that diversity alone does not differentiate AD from controls.

Differential abundance analysis identified five bacterial genera with significant alterations in AD patients. Overrepresented in AD, *Escherichia-Shigella* is associated with pro-inflammatory effects that may exacerbate neuroinflammation via the gut-brain axis. Conversely, the underrepresentation of *Adlercreutzia* suggests reduced anti-inflammatory capacity in AD. At the same time, the reduction of the *Mucinivorans* and *Hafnia* genera indicates a weakened gut barrier, supporting theories that inflammation and gut integrity shifts are relevant in AD pathology.

ML models validated with Leave-One-Out Cross-Validation LOOCV achieved the highest performance, yielding an AUC of 0.88 with Recursive Feature Elimination and Logistic Regression for feature selection combined with AdaBoost. This approach optimized the G-mean for balanced performance, which is essential for our sample's data imbalance, and demonstrated accuracy and robustness in capturing AD-related microbial patterns. In comparison, a 10-fold Cross-Validation method with SelectFromModel using Lasso regularization and a RandomForest classifier yielded an AUROC of 0.83, comparable to other studies that integrate functional microbiome and clinical data, suggesting that functional microbiome data could enhance model accuracy further.

Our findings support a role for gut microbiota in AD, with specific bacterial shifts potentially acting as markers for AD risk. Future studies could improve diagnostic accuracy by integrating functional microbiome and clinical data in larger cohorts, refining the use of microbial markers in AD diagnostics. These insights advance gut-brain axis research in neurodegeneration, with potential implications for early AD detection and therapeutic strategies.

Finally, the relatively small sample size of 175 participants, particularly the imbalance between AD and control groups, could limit the power and generalizability of the findings.

Also, the lack of longitudinal data restricts our ability to assess causal relationships between microbial shifts and AD progression. The reliance on taxonomic features alone, without incorporating functional microbiome data such as metabolites or gene expression, may overlook essential factors influencing AD. Furthermore, although ML models demonstrated promising results, they might be sensitive to biases inherent in the sample, such as overrepresenting specific demographics.

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