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Association of Gut Microbiota Composition with Alzheimer's-Like Disease in A Rat Model

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Association of gut microbiota composition with Alzheimer's-like disease in a rat model

A Thesis Submitted

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Declaration of Authorship

I, Hana Heiba, declare that this thesis titled, "***Association of gut microbiota composition with Alzheimer's-like disease in a rat model***" and the work presented in it are my own. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at thisUniversity.
- Where any part of this thesis has previously been submitted for a degree or any otherqualification at this University or any other institution, this has been clearly stated.
- Where I have consulted the published work of others, this is always clearly attributed.

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Abstract

With many countries turning 'grey' and facing an issue with aging populations, the risk of developing one form of neurodegenerative disease is increasing. Dementia, being the most common syndrome resulting from neurodegeneration, severely affects memory and cognitive functions. Alzheimer's disease (AD) is the most common neurodegenerative disorder, with an estimated 615,000 new cases will be added to the existing 5.7 million by 2030 (Alkasir et al., 2017).

In the current study, we establish a sporadic AD-like rat model by injecting STZ intracerebrally. Stool samples were collected at two time points; after three weeks for the acute stage, and 3 months for the chronic stage. Behavioral results show a significant difference in the working memory of the acute and chronic STZ-induced sAD-like rats when compared to age matched control groups. 16S rRNA sequencing on 18 samples revealed that Alpha diversity showed a significant difference between the STZ-induced sAD-like groups and control groups (p-value <0.005). Principal component analysis showed minimal clustering, and logistic regression did not show any significant differences in the abundance of bacteria across the samples.

In conclusion, this project did not align with the reported literature in all aspects, although there was a decrease in the Faith PD alpha diversity index, no significant differences were observed in the beta diversity index and relative abundance of bacteria among the groups. That could be due to the small number of samples used within each group.

Future work should increase sample size to produce statistically significant results. In addition, it is advisable to supplement the fecal samples collected from our experimental groups with CSF to add biomarker detection to correlate with bacterial taxa discovered.

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AD	Alzheimer's disease
sAD	Sporadic Alzheimer's disease
Aβ42P	Amyloid-beta 42 protein
GF	Germ-free
SCFA	Short chains fatty acids
APP	Amyloid precursor protein
PSEN1	Presenilin 1
PSEN1	Presenilin 2
CNS	Central nervous system
TNF-a	Tumor necrosis factor alpha
CRP	C-reactive protein
IL-1b	Interleukin 1 B
GI	Gastrointestinal
5HT	5-hydroxytryptamine
BBB	Blood-brain-barrier
ENS	Enteric nervous system
ANS	Autonomic nervous system
HPA axis	Hypothalamic-pituitary-adrenal axis
LPS	Lipopolysaccharide
NFkB	Nuclear factor kappa B
LBP	Lipopolysaccharide binding protein
EECs	Enteroendocrine cells
TLR	Toll-like receptors
SPI1	Spi-1-proto-oncoegene
CSF1R	Colony stimulating factor 1 receptor
CRF	Corticotropin-releasing factor

AS	Alpha synuclein
NO	Nitric oxide
STZ	Streptozotocin
ICV	Intracerebroventricularly
ATP	Adenosine -triphosphate
ChAT	Choline acetyltransferase
AChE	acetylcholinesterase
WM	Working memory
aCSF	artificial cerebral spinal fluid

Chapter 1

Introduction and Literature review

1.1 Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disorder that affects mental ability development and disturbs neurocognitive functions. AD is mostly characterized by protein misfolding, which leads to protein aggregation that results in neuronal loss (Hardy & Higgins, 1992; Selkoe, 2011). Certain conditions, such as cardiovascular diseases, hypertension and diabetes increase the risk of developing AD (Srivastava et al., 2021).

1.1.1 Types of Alzheimer's Disease

AD slowly but surely robs its victims of their most basic human qualities: memory, reasoning, abstraction, and language (Selkoe, 2011). One of the main challenges of neurodegenerative disorders is that by the time patients present with clinical symptoms, the progression of their disease is too far along. AD is no different, with molecular changes occurring in the brain 15-20 years before the first symptoms are observed in patients (Cerovic et al., 2019; Pritchard et al., 2017; Selkoe, 2011). There are two types of AD: the sporadic form with an average onset age of 80 years and the familial form which has a much younger onset age (Masters et al., 2015). The familial form of AD constitutes less than 1-5% of confirmed AD diagnoses (Pritchard et al., 2017) and is mostly associated with mutations in the amyloid precursor protein (APP), Presenilin 1 (PSEN1), and Presenilin-2 (PSEN2) genes in an autosomal dominant inheritance pattern (Kowalski & Mulak, 2019).

1.1.2 Histological features of Alzheimer's Disease

That the most prominent histological feature of AD is the formation of amyloid-beta 42 protein (Aβ42P) plaques and neurofibrillary tangles composed of hyperphosphorylated tau protein (Selkoe, 2011). Aβ is a transmembrane protein whose function is still unknown, that is derived by the proteolytic cleavage of amyloid precursor protein (APP) by a complex family of enzymes (γ secretases and βsecretases) (Masters et al., 2015). Aβ42 has two extra hydrophobic amino acids (Ala and Ile) at its carboxyl terminus unlike the more abundantly generated Aβ40 peptide (Selkoe, 2011). Tau is an intracellular microtubule-binding protein. When tau is hyperphosphorylated it results in the disassembly of microtubules leading to impairment in the axonal transport severely affecting the synaptic functions (Barbier et al., 2019).

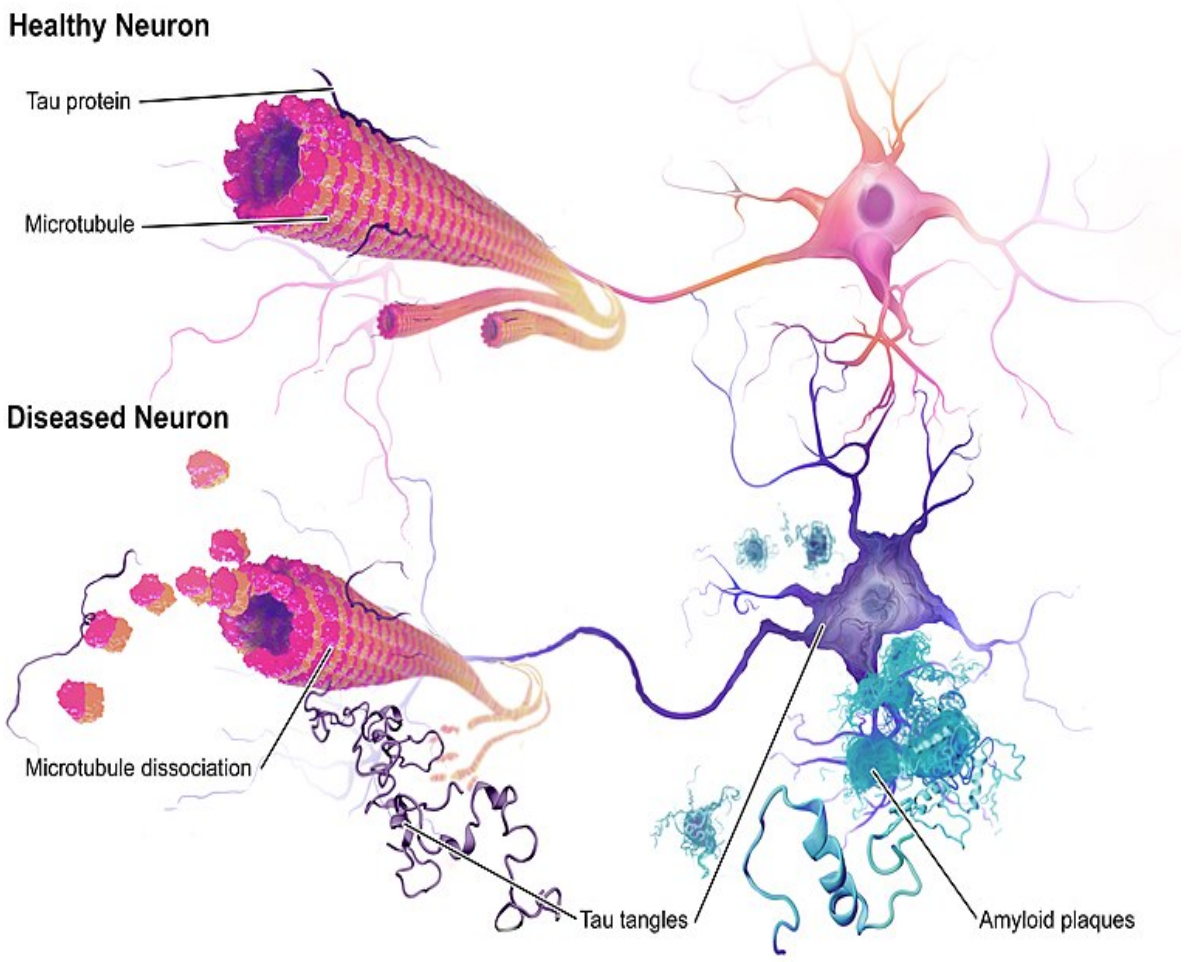

Figure 1 An illustration of the two main hallmarks of Alzheimer's disease. Hyperphosphorylated tau protein disassociates from microtubules causing them to disassemble and the phosphorylated tau aggregate to form neurofibrillary tangles. Amyloid plaques are composed of amyloid-beta 42 protein (Aβ42P) and has two extra hydrophobic amino acids (Ala and Ile) at its carboxyl terminus compared to the more abundantly generated Aβ40 peptide. The two extra amino acids in Aβ42 polypeptide makes it hydrophobic, which results in accumulation of the amyloid plaques. Source: creative commons license https://commons.wikimedia.org/wiki/File:Alzheimers_Disease.jpg

1.1.3 Inflammatory Response

The presence of the Aβ42P plaques and the hyperphosphorylated tau protein induce inflammation in the brain, leading to the stimulation of microglial cells (Abbayya et al., 2015). Microglial cells, in low concentrations, play a primary role in the neuroprotective function by removing Aβ42P plaques as well as other exogenous pathogens (Cerovic et al., 2019). In a sense, microglial cells maintain homeostasis by acting as mononuclear phagocytes against any noxious injury within the central nervous system (CNS) (Abbayya et al., 2015). Several factors such as age and genetic disposition compromise the function of glial cells. when their function is compromised, the chronic inflammatory response within the CNS persists. Being subjected to systemic inflammatory signals, glial cells start to release neurotoxic substances (Abbayya et al., 2015). This in turn contributes to the pathogenicity of AD. When microglial cells encounter infection or brain damage, they are "activated" and once the harmful stimulus is eliminated, they should revert to their resting state. However, in the case of AD microglial cells remain active (Cerovic et al., 2019). Activated microglial cells induce the production of proinflammatory molecules such as TNF-alpha, C-reactive protein (CRP), and IL 1B (Abbayya et al., 2015). These proinflammatory markers have been found to stimulate the production of Aβ42P and P-tau protein while Aβ42P and P-tau stimulate the production of proinflammatory molecules (Abbayya et al., 2015). In addition, activated microglial cells lose their phagocytic ability thus removal of Aβ via microglial cells decreases (Cerovic et al., 2019).

1.2 The Gut Microbiota

1.2.1 Role of the Gut Microbiota

The GI tract is home to the largest reservoir of microbes in the human body, with around 100 trillion microbial cells, distributed over 1,000 species many, of whom are anaerobic bacteria (Shreiner et al., 2015; Tremaroli & Bäckhed, 2012). The human gut microbiome can be divided into four major phyla; Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria, with more than 90% of relative abundance being composed of Firmicutes and Bacteroidetes and the remaining 10% represented by Actinobacteria and Proteobacteria (Binda et al., 2018).

Several studies have shown that the gut microbiota has an indispensable role in maintaining host homeostasis and metabolism such as vitamin production and absorption (Binda et al., 2018; Ramakrishna, 2013), breaking down substances that are otherwise indigestible by the host (Binda et al., 2018; Shreiner et al., 2015) and immune system development (Alkasir et al., 2017; Binda et al., 2018).

Rare strains of *Bacteroidetes ovatus* have recently been implicated in the breaking down of xyloglucans (XyG), a hemicellulose commonly found in plant cell walls, for the host to absorb (Larsbrink et al., 2014). Although the *B. ovatus* strains that have the ability to break down XyG are rare, Larsbrink et al showed that among 250 adult humans, 92% had at least one strain capable of digesting XyG, indicating the importance of these bacteria to human energy acquisition. Germ-free mice (GF), strains of mice that can be modulated to host-specific species of bacteria or whole communities, have been extensively used to study how the gut microbiota interacts with host physiology.

Various studies show that in these mice, the gut microbiota interferes with immune development response, bone-mass density modulation, and promotion of fat storage (Tremaroli & Bäckhed, 2012). Genetically obese mice (ob/ob), who are unable to produce

the appetite regulator leptin were found to have more Firmicutes and fewer Bacteroidetes than normal wild-type mice (Tremaroli & Bäckhed, 2012). Similar patterns have been observed in humans, where levels of *Prevotella* were negatively correlated with energy intake and adiposity (Furet et al., 2010; Tremaroli & Bäckhed, 2012). In addition, microbial genes encoding for enzymes responsible for the breakdown of carbohydrates, sugars, and short chains fatty acids (SCFA) were elevated (Ramakrishna, 2013). Decreased SCFA has been reported in neurodegenerative disorders such as AD (Komanduri et al., 2022). When gut microflora was taken from ob/ob mice and inoculated in germ-free lean mice, the germ-free mice developed obesity, suggesting that the gut microflora composition plays a role in obesity (Ramakrishna, 2013).

Through our understanding of various studies utilizing 16S sequencing, we know that *Bacteroidetes* and *Firmicutes* (Bonaz et al., 2018; Shreiner et al., 2015) are the dominant phyla found in the GI tract, with smaller amounts of *Proteobacteria* (Ramakrishna, 2013), *Actinobacteria*, and *Verrucomicrobia* (Bonaz et al., 2018; Ramakrishna, 2013). *Firmicutes* belonging to the genera *Clostridium clusters IX* and *XIV* such as *Eubacterium*, *Faecalibacterium*, *Roseburia*, and *Ruminococcus* are prominent members (Ramakrishna, 2013). While *Bacteroidetes* include bacteria belonging to the genus *Bacteroides* and *Prevotella. Bifidobacterium* is the major genus observed belonging to the phylum Actinobacteria (Ramakrishna, 2013).

1.2.2 Gut-Brain Access

The genius of the human body lies in the fact that every system, every organ, and every cell give signals to communicate with the rest of the body. The same is true when we talk about the gut-brain axis. This is a two-way communication port that connects the gut tract and the central nervous system. There are four main neuro-chemical pathways by which the gut microbiome and the central nervous system communicate.

First, a metabolic pathway where neuroactive compounds (neurotransmitters and neurotoxins) such as short-chain fatty acids (SCFA), serotonin (5HT), acetylcholine, tryptophan, and D-lactate and ammonia are released by the gut microbiome and transmitted through systemic circulation. These metabolites then cross the blood-brain barrier (BBB) where they modulate neural activity (Guo et al., 2021; Hung et al., 2022).

Second, a neural pathway where the enteric nervous system (ENS) and central nervous system (CNS) communicate through the Vagus nerve and the autonomic nervous system (ANS). Activation of the ENS is influenced by signals from the gut microbiome. These signals can directly influence gut cells, and regulate anti-inflammatory response of the peripheral immune system (Alkasir et al., 2017; Hung et al., 2022; Westfall et al., 2017).

Third,, an immune pathway where immune modulation is stimulated through pro-inflammatory cytokine release by the gut microbiome such as interleukin-1, interleukin-6 and tumor necrosis factor-alpha (Alkasir et al., 2017; Hung et al., 2022; Westfall et al., 2017).

Fourth, an endocrine pathway through the hypothalamic-pituitary-adrenal axis (HPA axis) (Alkasir et al., 2017; Hung et al., 2022; Westfall et al., 2017).

Metabolic pathways: Systemic circulation of neuroactive compounds through the BBB

Many of the gut microbes release compounds that can penetrate the BBB. *Lactobacillus* are known to release SCFA and acetylcholine. *Bifidobacterium* species also have the ability to produce SCFA, while *Escherichia, Bacillus, and Saccharomyces spp* produce norepinephrine (Borre et al., 2014). SCFA can translocate from the colonic mucosa to systemic circulation and penetrate the BBB, effectively affecting CNS functions (Cerovic et al., 2019).

Lipopolysaccharides (LPS), a common bacterial metabolite, can induce a strong immune response due to its interaction with CD14 and TLR4-MD-2 complex and a downstream responses lead to the activated of nuclear factor kappa B (NF-kB) (Bonaz et al., 2018; Guo et al., 2021). NF-kB has been incremented in AD pathogenesis due to its subsequent activation of pro-inflammatory cytokines (Guo et al., 2021). Furthermore, LPS plasma levels were elevated in AD patients and a positive correlation was found between high LPS levels and increased levels of blood monocytes/macrophage activation (Guo et al., 2021). LPS was also found to be colocalized with Aβ1-40/42 in amyloid plaques as well as traces of LPS was found in the hippocampus and superior lobe neocortex in AD patients (Guo et al., 2021).

Similar findings were observed in animal models; Chen et. al reported elevated serum levels of lipopolysaccharide-binding protein (LBP) in ADLP^{APT} mice (transgenic mice that exhibit AD pathology). This points to a probable systemic inflammation that is caused by gut-microbe released antigens including LPS into the circulatory system of ADLP^{APT} mice causing a permeable gut barrier and thus contributing to the AD pathogenesis.

Neural pathways: Enteric nervous system (ENS) and CNS communication through the Vagus nerve and the autonomic nervous system (ANS).

The ANS (sympathetic and parasympathetic branches) is responsible for the giving and receiving of signals from the lumen through enteric, spinal, and vagal pathways to CNS and from the CNS to the intestinal wall (Carabotti et al., 2015). Many of the GI key functions are controlled by the ANS such as gut motility and fluid handling, bicarbonate and mucus production, epithelial fluid maintenance, bile secretion and the mechanical distortion of the mucosa (Cryan et al., 2019). Incoming signals from the gut through the ANS are processed by the CNS and are then directed appropriate responses (Cryan et al., 2019).

The Vagus nerve is the 10th cranial nerve, it has the fastest and most direct route that connects the brain and the gut. The Vagus nerve is composed of almost 80% afferent fibers and 20% efferent fibers. This allows it to receive signals from the gut (bottom-up) and in turn send signals from the brain to the visceral (top-down). This nerve's afferents are laden with multiple receptors that it is called polymodal, in the sense that they respond to a variety of signals such as mechanical, hormonal, and chemical signals (Cryan et al., 2019). Vagal afferents interact with enteroendocrine cells (EECs) to detect bacterial metabolites either directly through the release of serotonin (5-hyroxytryptamine) or through or gut hormones such as cholecystokinin where receptors of these molecules are located on vagal afferents (Bonaz et al., 2018; Haas-Neill & Forsythe, 2020). Some interactions from the microbiota occur directly such as the case with butyrate, a long fatty-chain acid, that acts directly on afferent terminals. TLR4 are expressed on the afferent fibers of the Vagus nerve, toll-like receptors (TLR) sense bacterial metabolites such as LPS (Bonaz et al., 2018).

To further attest that the gut microbiome does in fact activate vagal sensory neurons, Goehler et al. were able to assess the levels of c-Fos, a neuronal activation marker, to test whether mice inoculated orally with the bacterium *Campylobacter jejuni* (*C. jejuni*) had elevated levels when compared to mice fed saline. To make sure that the ingestion of *C. jejuni* indeed triggered the vagal response before the immune signaling, they also tested for circulating cytokines. It was found that mice inoculated with the bacterium had a significant increase in the c-Fos levels in neurons bilateral to the vagal ganglia with no significant increase in circulating cytokines (Goehler et al., 2005).

Immune modulation through pro-inflammatory cytokine release by the gut microbiome

The gut hosts the largest concentration of immune cells in the body. To help maintain the homeostasis of the body from the countless of pathogens inhabiting this cavity, the

epithelium secretes a protective mucus layer that limits the interaction between host cells and microbial cells (Cryan et al., 2019). Different cell types inhabit the gut epithelium layer such as enterocytes, which express innate immune receptors and can secrete cytokines and chemokines, and chemosensory cells (Cryan et al., 2019).

Microglia constitute 5-12% of all brain cells and are highly versatile (Cryan et al., 2019). They can release cytokines, chemokines, regulate neurotransmitters, and even change their morphology once they have been activated. Cytokines play an integral role in maintaining homeostasis, these molecules recruit other immune cells to sites of infection (Cryan et al., 2019). They are classified as pro-inflammatory or anti-inflammatory (Cryan et al., 2019). Until recently, not much was known about the interaction between gut microbiome and the immune cells inhabiting the brain. However, rising evidence now show that having a diverse GI microbiota is in fact vital to the development of microglia in terms of maintenance and maturation (Cryan et al., 2019) and in instances were gut microbiota is compromised such as GF model animals the development of microglia was severely impacted (Cryan et al., 2019). Microglial cells can recruit monocytes from the periphery to the brain (Cryan et al., 2019), changes in the gut microbiota have been associated in the increase of activated monocytes in peripheral circulation, which then can be recruited to the brain via microglial cells (Haas-Neill & Forsythe, 2020). In GF animal models, microglia were found to be immature. Many microglial transcription and survival factors, such as SPI1 and CSF1R are downregulated in normal adult mice but were found to be upregulated in GF adult mice indicating that gut microbiota indeed plays a role in the maturation of the immune system (Cerovic et al., 2019; Matcovitch-Natan et al., 2016).

Endocrine pathway: Hypothalamus-Pituitary-Adrenal axis

The microbiota helps regulate the hypothalamus-pituitary-adrenal (HPA) axis activation state. The HPA axis is a stress efferent axis that manages stress response. Being part of the limbic system, it is involved in memory and emotional responses (Carabotti et al., 2015). Systemic pro-inflammatory cytokines activate the HPA axis through the release of

corticotropin-releasing factor (CRF). CRF is secreted from the hypothalamus and stimulates the adrenocorticotropic hormone from the pituitary gland which eventually leads to cortisol release from the adrenal glands (Carabotti et al., 2015). Being one of the major stress hormones in the body, cortisol affects many organs in the body including the brain. The release of cortisol due to HPA activation, in turn, governs the activation state of microglial cells in the brain. Cytokine release and monocyte recruitment to the brain from the periphery are also affected (Alkasir et al., 2017).

The HPA axis is a prime example of how neural and hormonal lines of communication are brought together to influence the gut cells such as immune cells, epithelial cells, enteric neurons, smooth muscle cells, and enterochromaffin cells (Carabotti et al., 2015). At the same time, these same cells are under the influence of the gut microflora bringing forward the idea of the microbiome-gut-brain axis.

Several studies have shown an association between dysbiosis of the gut with stress, anxiety, and depression (Foster & McVey Neufeld, 2013; Naseribafrouei et al., 2014), gastrointestinal disorders such as irritable bowel syndrome (Quigley, 2018). The result of this dysbiosis determines changes in intestinal motility and permeability (Foster & McVey Neufeld, 2013).

The use of different experimental approaches helped explore the contribution of microbiota in modulating the gut-brain axis. Such approaches include germ-free animals (GF), probiotics, antibiotics, and infection studies (Carabotti et al., 2015). Bacterial colonization was shown to be integral for ENS and CNS development in GF animals. The absence of gut bacteria was associated with an altered expression and turnover of neurotransmitters in the nervous system (Carabotti et al., 2015). In addition, sensory-motor functions such as delayed defecation and bowel transit time, and enlarged cecal size were also observed. Treating GF animals with species-specific bacteria, these changes resorted to normal functions (Carabotti et al., 2015).

1.3 AD and the Gut Microbiota

APP is regularly expressed in the ENS by enteric neurons and glia, thus supporting the idea that the ENS is somehow involved in AD pathogenesis (Chalazonitis & Rao, 2018; Kowalski & Mulak, 2019). Animal models of familial AD show that there is an accumulation of Aβ in the animal's plexuses but that did not result in dysmotility or neuronal density however, gut macrophages and pro-inflammatory were significantly higher than in control groups (Chalazonitis & Rao, 2018) indicating that the Aβ deposition triggers an inflammatory response leading to macrophage recruitment (Chalazonitis & Rao, 2018). APP/PS1 mice (double transgenic mice that express chimeric mouse/human APP protein and a mutant human presenilin 1) showed reduced brain Aβ deposition when treated with probiotics as well as changes in the circulating peripheral cytokines, chemokines and gut hormones (Kim et al., 2020). Furthermore, germ-free APP transgenic mice showed Aβ deposition upon transplanting altered microbiota indicting the role of gut microbiota in AD pathogenesis (Kim et al., 2020). Additionally, when WT fresh fecal matter was regularly fed to ADLP^{APT} (AD-like pathology with amyloid and neurofibrillary tangles transgenic) mice the Aβ and tau tangles depositions were controlled when compared to WT controls. Memory deficit and reactive gliosis were also reduced in ADLP^{APT} mice (Kim et al., 2020).

As mentioned, the gut is home to many types of pathogenic and symbiotic bacteria, some of which produce the bacterial version of amyloid proteins. *E. coli* produces curli, which helps in the formation of biofilms protecting the bacteria from physical and/or immune destruction. The bacterial amyloids differ from human amyloids in their primary structure they, however, retain similarities in their tertiary structure (Kowalski & Mulak, 2019). The immune system could be 'primed' due to the exposure of bacterial amyloids leading to an enhanced response to the endogenous production of neuronal amyloids in the brain. Chen et al hypothesized that bacterial amyloids have the ability to cross-seed host amyloids such as alpha-synuclein (AS) and trigger protein misfolding. The presence of these misfolded proteins 'prime' the immune system to their presence and illicit a

substantially severe immune response (Chen et al., 2016; Guo et al., 2021). This was done by exposing aged rats and transgenic nematodes expressing human AS to curli producing E. coli while control groups either received a mutated curli producing E. coli or E. coli with an empty vector. Rats exposed to curli-producing bacteria had a higher AS deposition in their guts and brain, enhanced microgliosis and astrogliosis when compared with both control groups. Proinflammatory cytokines such as TNF and IL6, and TLR2 were also elevated in the brain (Chen et al., 2016).

Other by-products of bacterial presence include Lipopolysaccharide (LPS). LPS is a cell wall component in gram-negative bacteria. LPS has been reported to bind to Toll-Like Receptor 4 (TLR4). TRL4 is mainly found on microglia in the CNS and once activated it leads to the recruitment of pro-inflammatory cytokines such as TNF alpha, IL-1 beta, and nitric oxide (NO)(Zhao et al., 2019). Using behavioral tests, immunofluorescence, and ELISA, Zhao et al were able to study the effect of injecting bacterial LPS in the brain of mice. Their results show that LPS injection leads to cognitive impairment much like those observed in AD and sickness behavior. Microglia were also activated, and neuronal loss was recorded in the hippocampus. Similar results were reported by Hauss-Wegrzyniak et al. (Hauss-Wegrzyniak et al., 2000)

AD patients have a high rate of gastrointestinal comorbidities, such as vitamin deficiency, obesity, diabetes, constipation, and/or diarrhea (Westfall et al., 2017). It was suggested that manipulating the gut microbiota using probiotics could potentially alleviate the symptoms of these chronic diseases (Westfall et al., 2017).

1.4 Experimental approaches for Alzheimer's Disease

Experimental models of AD play an important role in our understanding of the disease's pathogenicity and in providing critical information to developing therapeutic approaches (Drummond & Wisniewski, 2017). The most common experimental approach used in AD are transgenic animals, mainly mice and rats, that overexpress human genes associated

with familial AD such as APP and PSEN1 (Drummond & Wisniewski, 2017). Transgenic AD models are obtained by introducing the desired human gene into a zygote and then selecting animals portraying the desired phenotype (Götz et al., 2018). Other methods to generate transgenic animal models rely on homology-directed recombination (HDR) mechanisms and the use of gene editing tools such as CRISPR and TALENs (Götz et al., 2018). Non-genetic AD models depend on external factors to mimic certain aspects of AD. Intracranial stereotaxic injection-based models, such as ones using brain lysates from transgenic mice or from human brain lysates have been used to induce tau pathology in the models (Bolmont et al., 2007; Clavaguera et al., 2009; Götz et al., 2018). These intracranial injection models are cheaper than developing a transgenic line and yields results in a timely manner (Götz et al., 2018).

As the theories surrounding the influence of the gut microbiota on brain and behavior evolve, the use of germ free animal models has become a significant tool to comprehend how gut composition can be utilized as a therapeutic agent (Luczynski et al., 2016). Studies have indicated that GF mice have a longer lifespan than conventionally reared mice; however, investigations have also suggested that the immune system development in these mice is impeded, with a lower or absent expression of certain T-cell receptors (TLR), decreased IgA secretion, and decreased proinflammatory cytokine levels (Luczynski et al., 2016). They are also leaner in shape than conventionally grown mice despite consuming more calories; that has been attributed to the loss of the bacterial metabolite SCFA (Mayer et al., 2015).

Germ free mouse models that also have genetic mutations of AD have been used as well. These models have been shown to have reduced amyloidosis and reduced glial reactivity (Cerovic et al., 2019). Other models utilize AD mutated mice and subject them to broad spectrum antibiotic to severely compromise their gut microbiota, such models have been reported to also reduce amyloidosis and glial reactivity (Cerovic et al., 2019).

1.4.1 STZ induced sAD-like rat model

Lannert and Hoyer first described a rat model for the study of long-term cognitive function, learning, and memory. They injected streptozotocin (STZ), a compound that is known to inhibit insulin receptor function directly in the cerebrospinal fluid in the cerebral ventricles using the intracerebroventricularly (ICV) method. STZ [2-Deoxy-2{[methyl-nitrosoamino)carbonyl] amino}D-glucopyranose (C8H15N3O7)] is a glucosamine-nitrosourea compound. When metabolized, STZ generates a cytotoxic product that destroys beta-cells in the pancreas and results in diabetes mellitus. STZ metabolites have alkylating properties that give rise to reactive oxygen species, therefore, resulting in oxidative stress and DNA damage (de la Monte et al., 2006; Zhang et al., 2020). Their results showed that ICV STZ injection-induced long-term changes in metabolism, memory, and cerebral glucose levels (Agrawal et al., 2009; de la Monte et al., 2006; Lannert & Hoyer, 1998). This model was deemed appropriate for the study of the human sporadic form of AD (Agrawal et al., 2009; Duelli et al., 1994; Ejaz Ahmed et al., 2013; Espinosa et al., 2013; Ishrat et al., 2009; Muller et al., 2017; Zhang et al., 2020).

The inhibition of adenosine triphosphate (ATP) and acetyl-CoA synthesis due to The ICV injection of STZ in a sub-diabetogenic dosage leads to prolonged dysfunction of brain metabolism, oxidative damage, and cognitive impairment. This leads to a cholinergic deficiency and a reduced choline acetyltransferase (ChAT) activity in the hippocampus (Ishrat et al., 2009; Reeta et al., 2017)and increased acetylcholinesterase (AChE) activity (Agrawal et al., 2009; Reeta et al., 2017). The injection of STZ in the brain causes a localized insulin insensitivity therefore not affecting the pancreatic insulin. Sporadic AD has sometimes been described as type 3 diabetes due to insulin insensitivity in the brain (de la Monte et al., 2006). Intracranial STZ injections have been recorded to increase amyloidogenesis, phosphorylated tau protein, limbic neuronal damage all of which are symptoms of sporadic AD (Agrawal et al., 2009; Espinosa et al., 2013; Zappa Villar et al., 2020).

1.5 Hypothesis

We hypothesized that STZ IVC injection will mimic symptoms of sAD. As such, the gut microbiota of acute STZ and chronic STZ groups will have lower diversity compared to age matched controls. We expect to see differences in the relative abundance of bacteria between the two types of groups (STZ injected and normal controls).

1.6 Aims of the study.

Aim 1: Establish an STZ-induced sAD-like rat model.

Aim 2: Perform 16S rRNA amplicon sequencing and analysis on pellets collected from our STZ-induced sAD-like rat model at two time points corresponding to acute and chronic disease stages.

Chapter 2

Materials and Methods

This project was conducted in collaboration with Nada Mostafa, a graduate student at the Biotechnology program, AUC. Rats were randomly divided into control and experimental groups. All procedures were performed in compliance with the national institute of health (NIH) guidelines for the Care and Use of Laboratory Animals. The procedures were approved by the institutional animal care and use committee (IACUC), Zagazig University (Approval number: ZU-IACUC/3/F/56/2020) in concordance with the ARRIVE guidelines (Moustafa, 2022).

2.1 STZ Rat Model establishment

Four groups were established for this project.

Group 1 Control acute (n=2, 1 male and 1 female), no experimental procedures were conducted on these rats to allow for basal comparison.

Group 2 STZ acute (n=6, 3 males and 3 females) were injected with a single dose of STZ (3mg/kg) in an artificial cerebral spinal fluid (aCSF) via intracerebroventricular (ICV) injection. Modified T-Maze and pellet collection took place three weeks after injection.

Group 3 Chronic STZ (n=6, 3 males and 3 females) were injected with a single dose of STZ (3mg/kg) in an artificial cerebral spinal fluid (aCSF) via intracerebroventricular (ICV) injection. Modified T-Maze and pellet collection took place 3 months after injection.

Group 4 Control chronic (n=4, 2 males and 2 females), no experimental procedures were conducted on these rats, memory tests and pellet collection took place after 3 months to account for the age difference for the chronic STZ group.

The two experimental groups followed the same STZ injection protocol while the memory testing time-point differed. Male and female Sprague-Dawley rats were anesthetized with intraperitoneal Ketamine/Xylazine mix (Ketamine 75 mg/kg and Xylazine 5mg/kg) (Flecknell, 2009). Intracerebral injection of Streptozotocin was performed according to the methods previously described by (Hau & Schapiro, 2002; Langhans et al., 2016). The animals were allowed to recover in clean cages with heating pads. Rats were closely monitored for signs of pain, infection, or excessive weight loss.

2.2 Modified T maze test

Cognitive testing was performed using a modified T maze test. This test evaluated the short-term (working memory). It is a simple test and puts minimal stress on the animals (Wu et al., 2018). The test is designed to take place in a device shaped like a T laid down horizontally (figure 2). Animals are placed at the end of the T and allowed to choose between the right or left arms of the T. During the second trial, rats typically choose the alternative arm, reflecting the memory of their first trial. Here, rats are utilizing their short-term working memory.

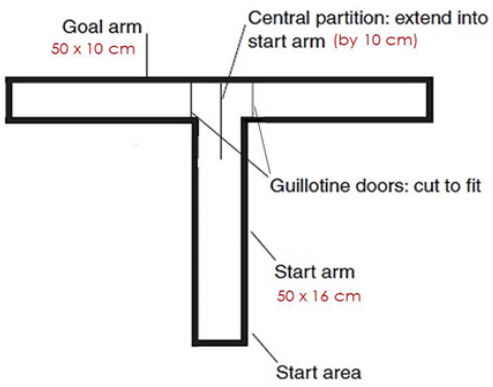

Figure 2 Illustration of the T-maze apparatus

2.3 Open maze test

The open maze test can be used to measure locomotion, anxiety, memory and exploratory behavior (Seibenhener & Wooten, 2015). Rats were placed in a 100 cm x 100 cm square wooden grey enclosed arena that is divided into 20 cm squares using a permanent marker creating central squares (squares not touching the sides of the arena) and peripheral squares (on the outer sides of the arena) (figure 3). Rats were placed in a corner square with their heads facing the corner. Each rat was observed for 5 mins, rearing frequency (vertical activity, both front limbs are off the floor) and number of squares crossed using the whole body (horizontal activity) were recorded. Rats were removed after 5 minutes and fecal pellets counted. The arena was cleaned before and after testing each subject (Deacon, 2006).

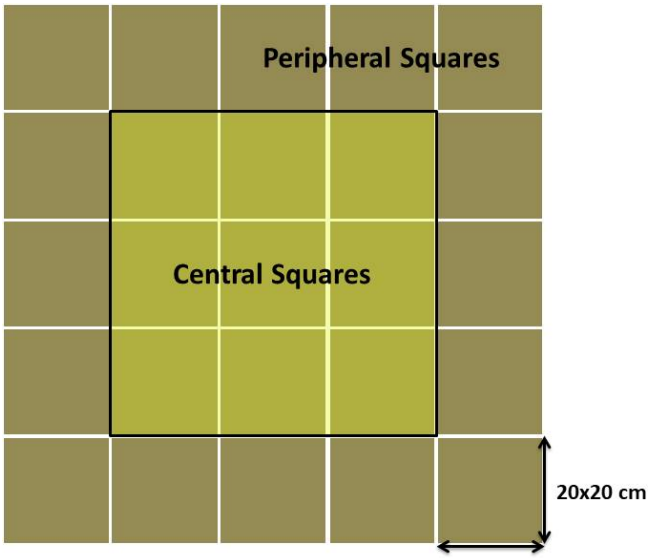

Figure 3 Schematic representation of the open field test arena dimensions.

2.4 Fecal pellet sample collection

Fecal pellet sample collection procedure was adapted from IDEXX Laboratories Inc. Briefly, Eppendorf tubes 1.5 ml were sterilized in an autoclave for 25 minutes and then placed in an oven at 90 C for 15 mins a day prior to sample collection. Rats were held by a handler while a sterile 1.5 ml Eppendorf was placed beneath the anus. The sample was

collected and placed on dry ice immediately. Samples were stored at -80C until processing.

2.5 DNA Extraction and Integrity Gels

DNA was extracted using Qiagen's DNeasy PowerSoil kit (CAT #47014) according to the manufacturer's guidelines. Fecal pellets (0.25 gm) were homogenized. Elution of DNA was done in 150 ul of pre-heated (at 56oC for 10 mins) elution buffer. Samples were run on a 1.5% agarose gel (40 minutes, 80V) to ensure DNA quality.

2.6 16S Sequencing using Illumina MiSeq

Sequencing was done at the 57357 hospital's genomics lab. Samples were sequenced according to the Illumina 16S rRNA protocol (Illumina, USA). The Illumina 16S rRNA protocol is PCR-based. There are two PCR amplification steps that are followed by clean-up steps. The initial amplification amplifies the V3-V4 region of the 16S rRNA gene using degenerate primers with overhang adaptors attached.

Forward primer used was: 5'
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG,
and

Reverse primer used was: 5'
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC.

Following this step, AMPure XP beads were used to purify the amplified V3 and V4 amplicons from free primers. Then Nuxtera XT Index kit (Illumina, USA) was used to

attach dual indices and Illumina sequencing adaptors to each sample. Clean-up using AMPure XP beads was done, followed by validation of library size using Bioanalyzer DNA 1000 chip with an expected size of ~630 bp. The library was then quantified, normalized, and pooled according to Illumina manufacturing instructions (Illumina, USA).

2.7 Data Analysis

Preprocessing and taxonomic identification were performed using DADA2 workflow, filter-And-Trim to filter and trim low-quality reads, and truncLen=c(290,270) and maxEE=c(8,9). Error rates were learned by nbases=90000. Filtered reads were merged and inferred using DADA2 functions DADA, mergePairs, and Chimeras were removed. Generated sequences table were imported to QIIME2. QIIME2 feature-classifier was used to extract reads from silva-138-99 database with the parameters (f-primer CCTACGGGNGGCWGCAG, r-primer GACTACHVGGGTATCTAATCC, min- length 350, max-length 580) and trained using fit-classifier-naive-Bayes.

The classification was performed by classify-sklearn. The tree was generated using "align to tree mafft-fasttree", and diversity metrics were calculated using core-metrics-phylogenetic: Kruskal-Wallis pairwise t-test was used to calculate statistical tests. QIIME2 was used to visualize the results in addition to R packages phyloseq and ggplot2 at a minimum depth of 25000.

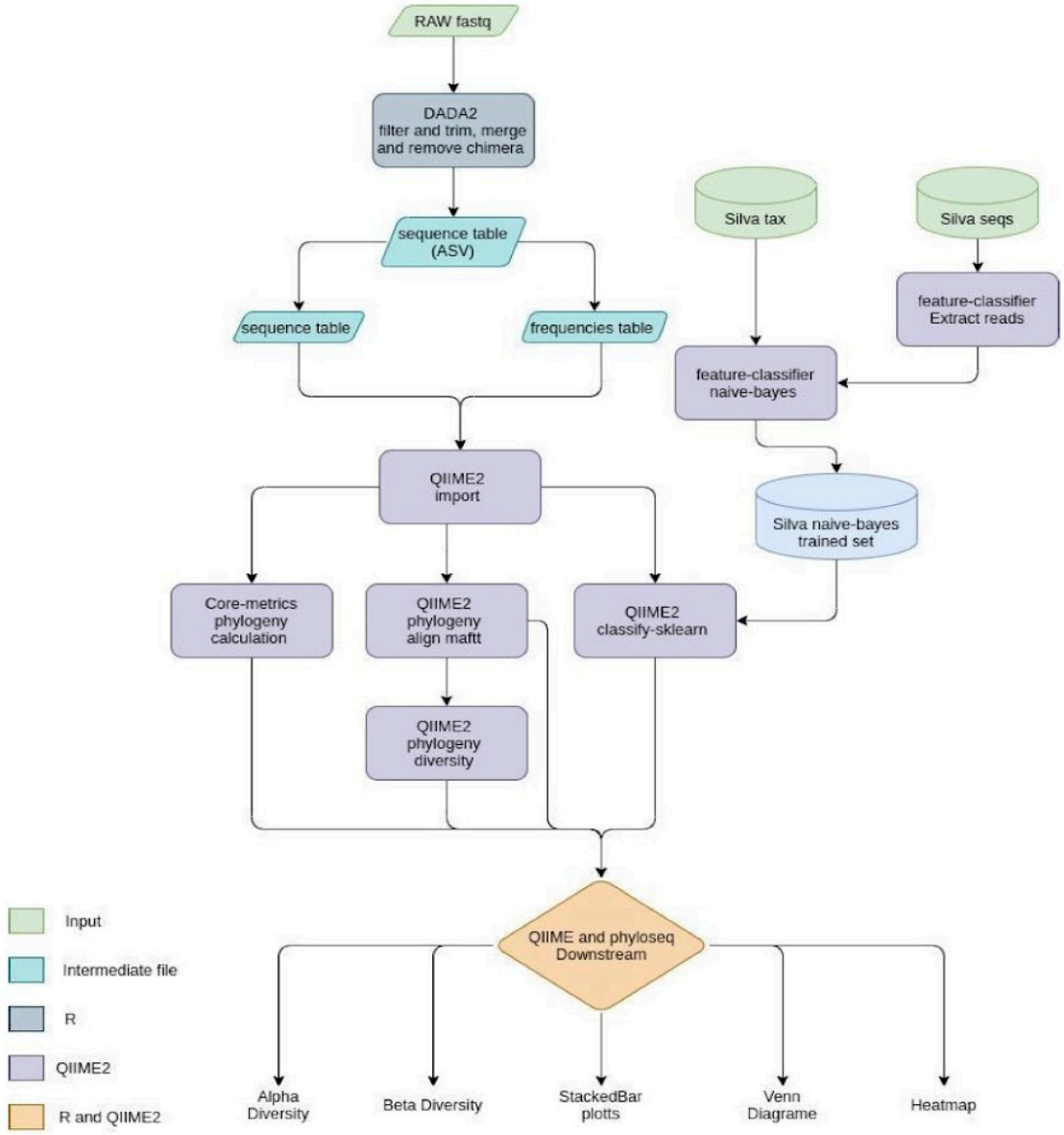

Figure 4 A schematic representation of the Data analysis workflow.

Chapter 3

3.1 Results and Discussion

3.1 Characterization of the STZ-induced sAD-like rat model.

The histological and behavioral characterization of the model was performed in collaboration with Nada Moustafa, Biotechnology program, AUC and were described in detail in her thesis dissertation (Moustafa, 2022).

3.1.1 Cognitive testing using Modified T-Maze test.

Normally, rats have the tendency to explore their surroundings, and so when placed in a T-maze they alternate between maze arms with every trial, this behavior demonstrates the working memory of their previous spatial location (Davis et al., 2017). We tested cognitive function using a modified T-maze spontaneous alternation test. There were significant differences (p-value>0.01) in the working memory (WM) in terms of reduction of alteration percentage between the control group and the acute STZ with a mean of 34.44±11.3 and chronic STZ with a mean of 28.89±7.82 groups. No significant difference was observed when the two STZ groups were compared together. Reduction in alteration rate reflects poor working memory and cognitive decline. Similar results were reported by Wu et al using a global cerebral ischemia rat model; the experimental group exhibited significantly less alteration rates when compared to control groups (Wu et al., 2018). Davis et al utilized a familial AD mouse model (3xTgAD mice) in a T-maze to measure short-term memory spatial performance; they report that 3xTgAD mice had an impaired short-term spatial memory due to the decrease in alternating rate between the two arms of the maze (Davis et al., 2017).

3.1.2 Assessment of locomotion and anxiety related behavior in an open maze test

STZ groups exhibited significantly lower locomotive activity in both vertical activity (p-value<0.05) (rearing frequency) and horizontal activity (p-value<0.001) (total number of squares crossed). No significant differences were seen among the two STZ groups together nor between the control groups. Higher horizontal activity shows less anxiety and increased locomotion and exploration behavior. (Zappa Villar et al., 2020) observed less crossing activity in Sprague Dawley rats treated with STZ. On the other hand, (Angelova et al., 2019) reported that Wistar male rats treated with STZ-IVC showed higher crossing and rearing rates compared to the control groups. they attributed that to decreased anxiety and abnormal habituation behavior. These discrepancies could be due to the different rat strains, sex of the animal, shape, and color of the open field test arena.

Assessing the number of fecal pellets and time spent in central squares showed only the chronic STZ group had a significantly higher number (p-value<0.05) of fecal pellets when compared to all other groups. However, both STZ groups spent significantly less time in central squares compared to the control groups. Zappa et al also reported decreased crossing activity in their model indicating lower exploratory behavior (Zappa Villar et al., 2020).

3.1.3 Histopathological Analysis

Sections of the hippocampus from STZ groups were stained by Hematoxylin and Eosin (H&E) stains or Cresyl Violet Staining (Nissl Staining). The acute control group showed normal cellularization while STZ groups showed signs of neurodegeneration and inflammation. Enlarged astrocytes were seen as well as degeneration of pyramidal cells,

neurofibrillary tangles and amyloid deposition (staining images not provided; please refer to Nada Alaa's thesis dissertation (Moustafa, 2022).

3.2 Analysis of 16S rRNA sequencing

3.2.1 Alpha diversity

Alpha diversity (intrasample diversity) using faith's phylogenetic diversity index showed a significant decrease ($p<0.05$) in both STZ acute and STZ chronic groups (Figure 5) compared to their controls which is consistent with the literature (Vogt et al., 2017). Faith's pd considers the phylogenetic relatedness of the samples and thus is considered a suitable index to measuring biodiversity (Armstrong et al., 2021). The alpha diversity calculated using Pielou's evenness index did not show any significant difference (Figure 6).

3.2.2 Beta-diversity

The principal component analysis, which depicts the beta diversity, did not show clustering of the groups. That could be due to the small sample size assigned to each group. The acute STZ control samples are always seen clustered together away from the chronic STZ control group and closer to the STZ acute group (Figure 7). While the chronic STZ control is seen near the STZ chronic group. That could be explained by the age factor, it may seem that age plays a larger role in the composition of bacterial colonization than the experimental procedure done. In addition, confounding factors such as stress induced from handling rats to excite defecation could influence the abundance and diversity of the gut microbiome (Xu et al., 2020). Zhan et al reported that SAMP8 mice (an accelerated aging model) clustered away from normal controls in both PCA and principal coordinate analysis indicating different gut microbiota composition (Zhan et al., 2018). Vogt et al also reported significant segregation in the PCA plots of AD patients gut microbiota compared to healthy controls (Vogt et al., 2017).

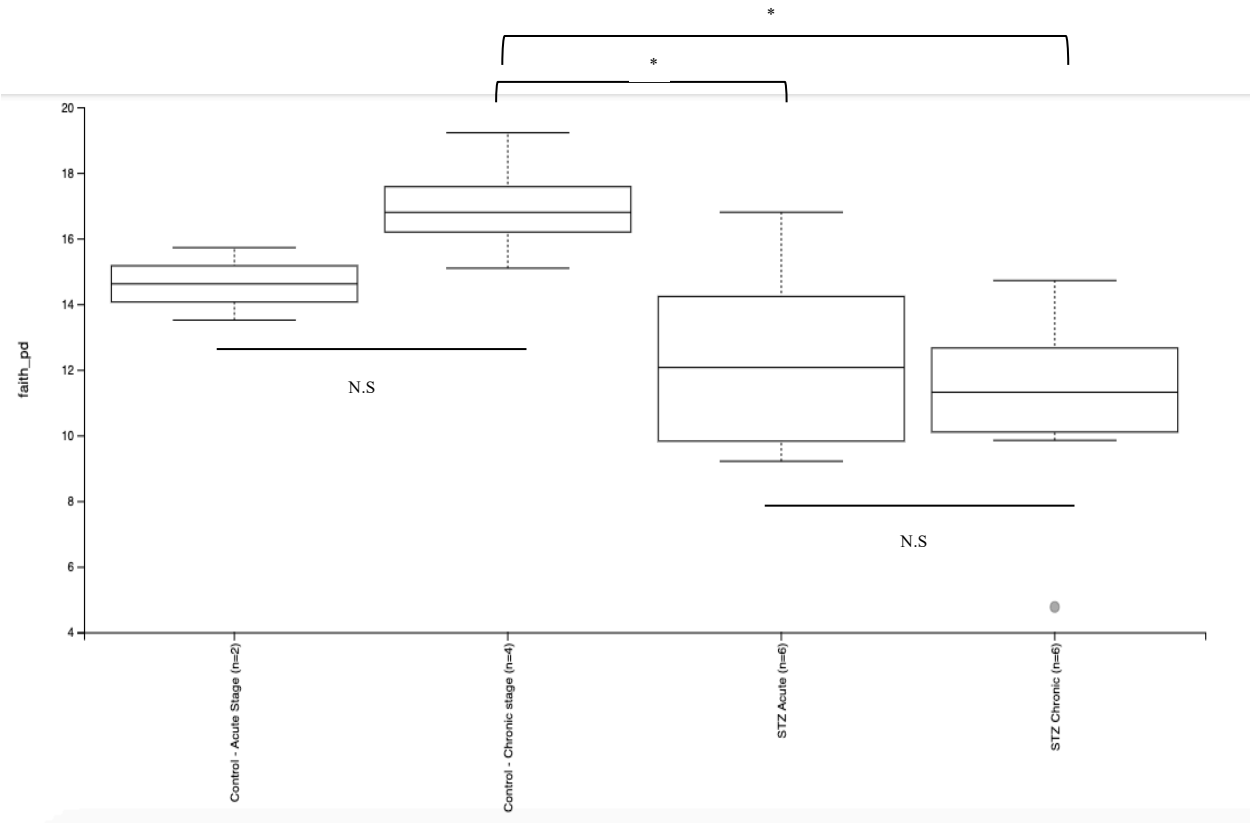

Figure 5 Alpha diversity using faith phylogenetic diversity index. Statistical difference was calculated using Kruskal-Wallis pairwise test between control acute, control chronic, STZ acute and STZ chronic. No significant difference was observed between the two control groups (p-value>0.05). No significant difference observed between the STZ acute and STZ chronic groups (p-value>0.05). A significant decrease in the alpha diversity was seen between the Control chronic and STZ acute (p-value <0.05) and STZ

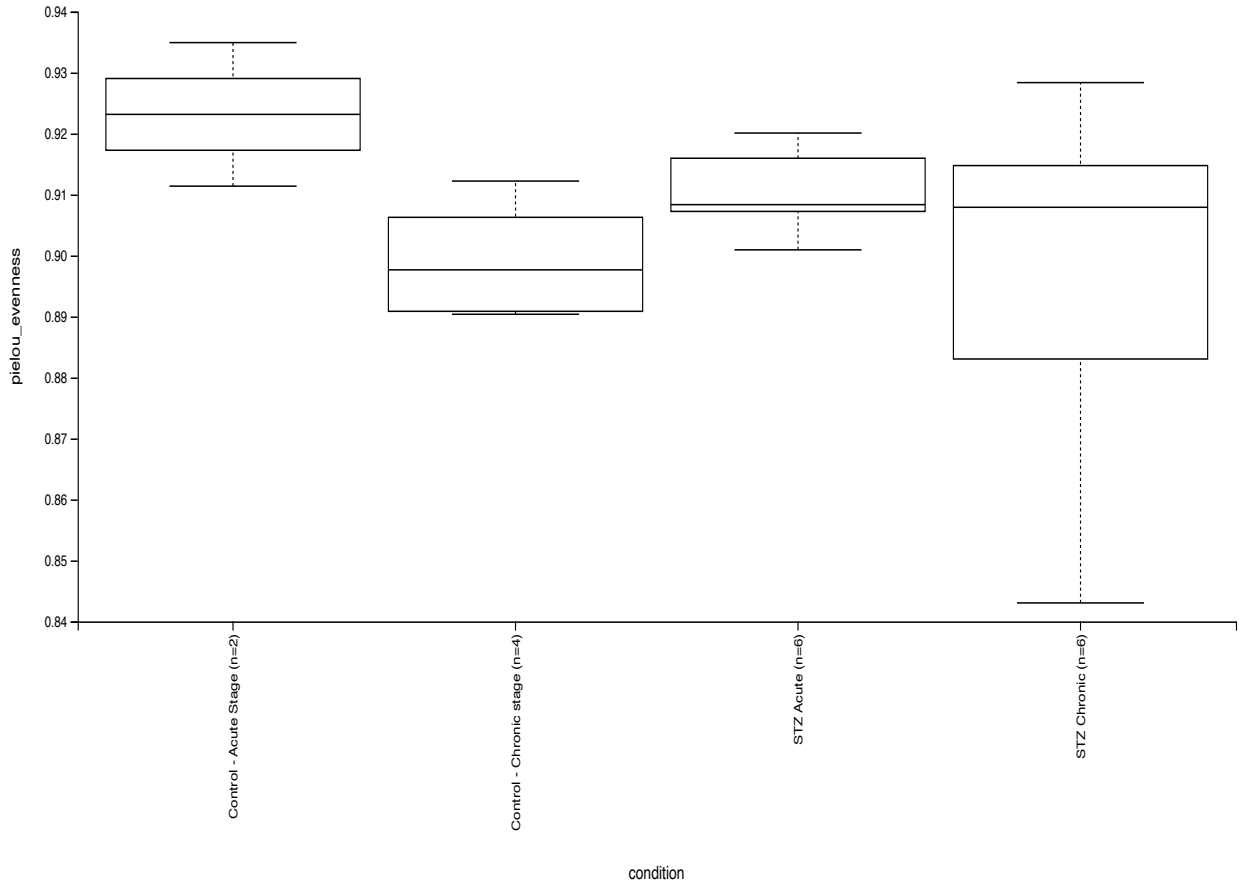

Figure 6 alpha diversity using Pielou's evenness index, statistical significance was calculated using Kruskal-Wallis pairwise test. No significant differences were identified across the four groups (p value>0.05 in all groups).

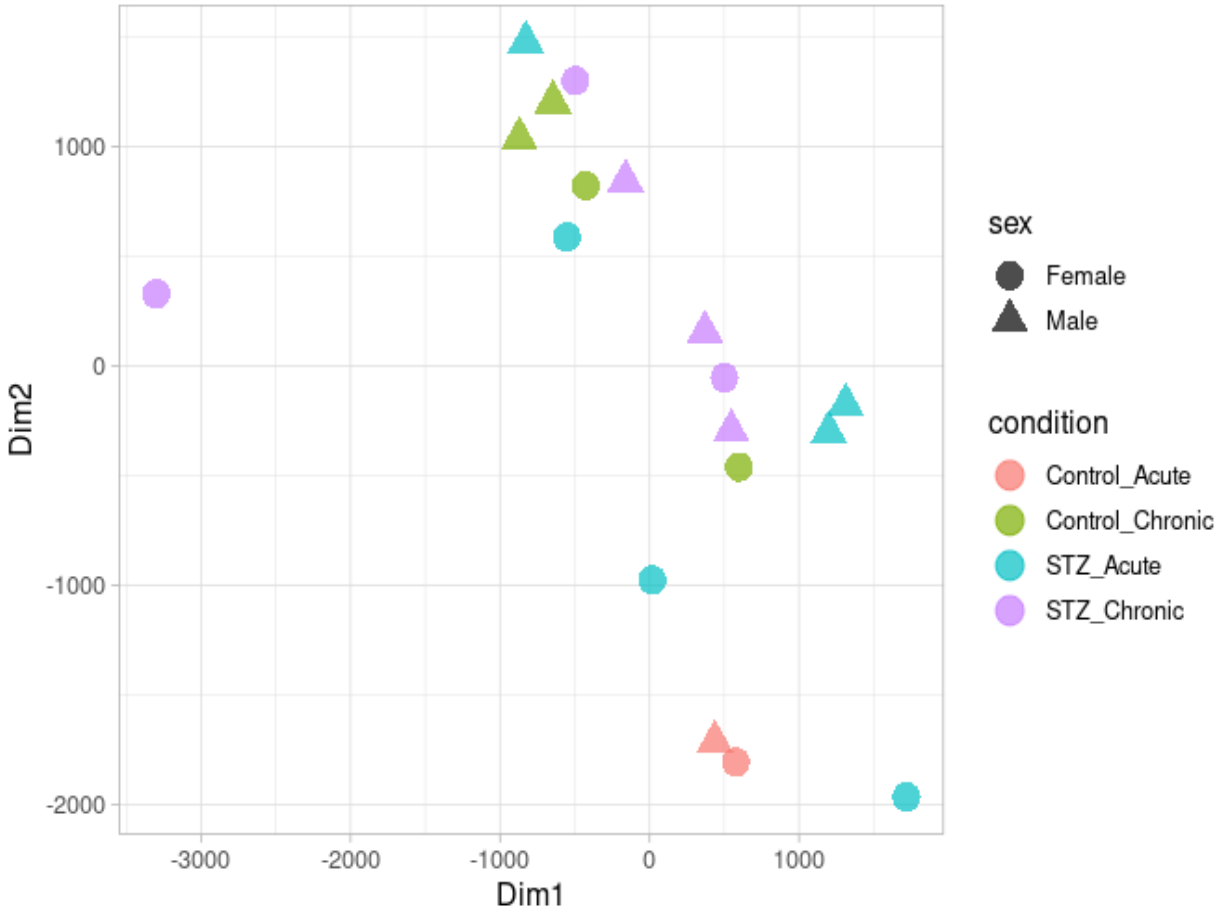

Figure 7 Principal component analysis. No significant clustering can be detected between the STZ groups and the control groups.

3.2.3 Relative abundance and regression analysis

Relative abundance of 43 genera of bacteria were detected in 18 samples. No differentially abundant genera were detected across the four groups (Figure 8). Regression analysis of the relative abundance did not show any significant changes of bacterial abundance across the samples. Logistic regression was used to calculate significant differences in the relative abundance of bacterial genera across the four groups. However, no differences were seen. That could be explained by the limited number of samples assigned to each group. Kim et al reported no significant changes in gut bacterial composition between wild-type mice and ADLP^{APT} mice. They suggested that community-level alteration, rather than specific taxa changes, may be associated with AD pathogenesis in ADLP^{ATP} mice (Kim et al., 2020).

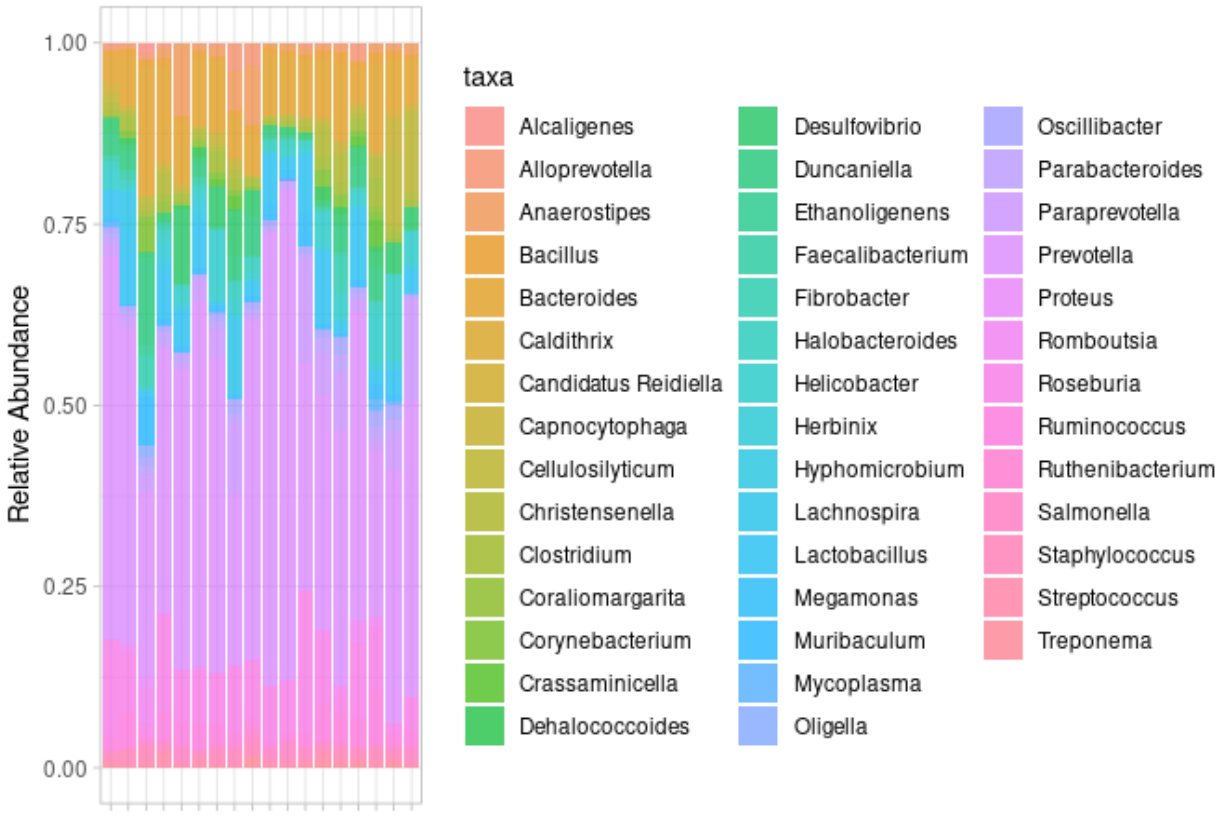

Figure 8 the relative abundance of the bacteria (genus level) in each sequenced sample. The predominant genus found across all samples was *Prevotella* followed by *Roseburia*, *Bacteroides*, and *Paraprevotella*. Logistic regression was used to calculate any significant differences of bacterial abundance between the group. No such difference was seen p-value>0.05.

Although we are not reporting compositional changes in the bacterial gut microbiome between our experimental groups and the control groups in this study, other authors have successfully found microbiome gut changes in their models be it human or animal. Vogt et al reported that the dominant phyla found in the 50 participants (AD n=25, control n=25) were Firmicutes (78%) and Bacteroidetes (15%) of total abundance. Actinobacteria were detected in lesser quantity (2.6%) followed by Verrucomicrobia (2.6%), and Proteobacteria (1.1%). AD participants had elevated levels of Bacteroidetes and lower abundance of Firmicutes and Actinobacteria. Within the Firmicutes; the families *Ruminococcaceae*, *Turicibacteraceae*, *Peptostreptococcaceae*, *Clostridiaceae*, and *Mogibacteriaceae*, and the genera *SMB53* (family *Clostridiaceae)*, *Dialister*, *Clostridium*, *Turicibacter*, and *cc115* (family *Erysipelotrichaceae*) were all less abundant in AD participants (Vogt et al., 2017). While the families *Gemellaceae,* the genera *Blautia*, *Phascolarctobacterium*, and *Gemella* were elevated. In the Bacteroidetes phylum; the families *Bacteroidaceae* and *Rikenellaceae* were elevated (Table 1) (Vogt et al., 2017).

Decrease in the phylum Firmicutes has been associated with individuals suffering from type 2 diabetes (Hung et al., 2022). Insulin resistance is implicated with increased amyloid deposition in asymptomatic middle-aged adults and decreased cerebral glucose metabolism hence increasing the risk of AD. These changes along with the microbial changes observed could lead to speculation that alteration in the gut may influence AD pathology through the promotion of insulin resistance and diabetes (Hung et al., 2022; Vogt et al., 2017). Similar results were reported by Harach et al with a general decrease in the Firmicutes phylum specifically the family *Allobaculum* showing significant reduction in abundance (Harach et al., 2017).

Vogt et al reported elevated Bacteroidetes in their study. This phylum contains numerous gram-negative genera that are elevated in patients with type 2 diabetes and Parkinson's disease such as *Bacteroide* (Vogt et al., 2017). Gram negative bacteria contain LPS as a major component to their cell wall. LPS can trigger inflammation response through the

release of pro-inflammatory cytokines by migrating from the gut to systemic circulation (Vogt et al., 2017). LPS plasma levels were elevated in AD patients, and a positive correlation was found between high LPS levels and increased levels of blood monocytes/macrophage activation (Guo et al., 2021). LPS was also found to be colocalized with Aβ1-40/42 in amyloid plaques as well as traces of LPS was found in the hippocampus and superior lobe neocortex in AD patients (Guo et al., 2021). In addition, postmortem brain tissue of AD patients showed LPS co-localized with gram-negative E. coli bacteria fragments and amyloid plaques. Therefore, an increase in gram-negative bacteria such as *Bacteroides* can potentially increase the translocation of LPS into systemic circulation and by default contribute to the AD pathology (Vogt et al., 2017). Similar results were reported by Harach et al in their APP/PS1 GF mouse model with the family *Rikenellaceae* being significantly higher in abundance compared to wild type controls (Harach et al., 2017). However, in a human cohort, *Rikenellaceae* was reported to be less abundant in AD patients compared to healthy controls (Hung et al., 2022). More discrepancies were reported by Hung et al where Chinese AD patients did not exhibit altered abundance while American AD patients did. Similarly, the abundance of *Alistipe* was increased only in American AD patients and not Chinese AD patients (Hung et al., 2022). These inconsistencies show that gut microbiota is influenced by many factors such as geographical location, diet, ethnicity and age (Hung et al., 2022).

Actinobacteria are generally considered 'good' bacteria, with many health benefits to their host (Binda et al., 2018; Vogt et al., 2017). Decrease in Actinobacteria specifically the genus *Bifidobacterium* were noted in AD patients. Species belonging to this genus have been implicated with anti-inflammatory properties and a decrease in gut permeability. Higher levels of *Bifidobacterium* through supplementation given to mice showed lower LPS levels and improved gut barrier properties (Binda et al., 2018; Vogt et al., 2017). It has also been documented that germ-free mice inoculated with human gut microbiota with elevated levels of *Bifidobacterium* exhibited reduced bacterial infiltration to systemic circulation (Vogt et al., 2017). Harach et al also reported reduced levels of Actinobacteria in APP/PS1 GF mice (Harach et al., 2017).

Verrucomicrobia, much like Actinobacteria, have a beneficial effect on their host (Geerlings et al., 2018). Studies show that decreased levels of Verrucomicrobia were reported in pre-diabetics and type 2 diabetes (Geerlings et al., 2018). Reduced abundance of Akkermansia, a family within the Verrucomicrobia phylum, was inversely associated with obesity and type 2 diabetes. Supplementation of Akkermansia through prebiotics restored fat-mass gain and reduced inflammation in APP/PS1 GF mice mouse model (Harach et al., 2017). In concordance with these accounts, high levels of Akkermansia were reported in athletes with low body mass index (Geerlings et al., 2018). Akkermansia spp were linked to strengthened gut-barrier function in mice and by that can influence metabolic endotoxemia (Geerlings et al., 2018; Harach et al., 2017). As such, one can suggest that decreased abundance of Akkermansia spp in AD models may help in disrupting the gut barrier and therefore, increase inflammatory response/events (Harach et al., 2017).

Table 1: Alteration in Gut Microbiota in AD/Dementia models.

Model	Phyla	Elevated in abundance Family Level	Lower in abundance Family Level	Elevated in abundance Genus Level	Lower in abundance Genus Level	Source
Humans	Firmicutes	*Gemellaceae, Phascolarctobacterium, Gemella*	*Ruminococcacea, Turicibacteraceae, Peptostreptococcaceae Clostridiaceae, Mogibacteriaceae, Clostridiacea, Erysipelotrichacea*	*Blautia, Phascolarctobacterium*	*SMB53, Dialister, Clostridium, Turicibacter, cc115*	(Vogt et al., 2017)
	Actinobacteria		*Bifidobacteriaceae*		*Bifidobacterium, Adlercreutzia*	
	Bacteroidetes	*Bacteroidaceae, Rikenellaceae*		*Bacteroides, Alistipes*		
	Proteobacteria			*Bilophila*		
$APP^{swe}/PS1\Delta^{E9}$ transgenic mice	Firmicutes		*Ruminococcus, Butyricicoccus*		*Butyricicoccus pullicaecorum*	(Zhang et al., 2017)
APP/PS1 GF mice	Bacteroidetes	*Rikenellaceae*				(Harach et al., 2017)
	Firmicutes		*Allobaculum*			
	Verrucomicrobia		*Akkermansia*			

Model	Phyla	Elevated in abundance Family Level	Lower in abundance Family Level	Elevated in abundance Genus Level	Lower in abundance Genus Level	Source
SAMP8 transgenic mice	Firmicutes		*Christensenellaceae, Ruminococcaceae*			(Zhan et al., 2018)
	Thermodesulfobacteriota		*Desulfovibrionaceae*			

Conclusion and Future works

Over the past decade, there has been mounting evidence suggesting a correlation between gut microbiome and cognitive diseases such as AD and Parkinson's. In this project we attempted to investigate the association of bacterial genera abundance to the pathology of AD using an STZ induced sAD-like rat model. We collected fecal samples at 2 stages (acute and chronic) from our AD model. Establishment of the rat model was validated using behavioral tests and histopathology (results not shown in the project; please refer to Nada Alaa's thesis project) followed by targeted 16S rRNA amplicons sequencing of eighteen samples.

Animal models have been a pivotal in experimental studies. They can be used to embody initial proteinopathy and pathological features found in many sporadic human diseases. These models can be modified to study the effect of certain genes on disease, development, and possible remedies for mankind. However, animal models cannot be expected to fully portray the complex pathways of human disease. They allowed us to refine the fields of medicine and pharmaceuticals by utilizing them to better our understanding of complex molecular and cellular mechanisms (Dawson et al., 2018).

Alzheimer's disease is one of the most perplexing neurodegenerative diseases, various animal models have been devised to help us understand the pathogenesis of AD. Some models are genetically based to replicate amyloid pathology. These model target gene precursors such as APP, PSEN1, and PSEN2 (Dawson et al., 2018). Other models, such as the one used in this project, rely on metabolic induced damage with external substances such as STZ (Zhang et al., 2020).

Despite the disappointing results of this project, multiple sources reported that AD patients were observed to have lower microbial diversity when compared to age and sex matched controls. Phyla of *Firmicutes* and *Actinobacteria* decreased while *Bacteroidetes* increased in abundance in AD patients (Vogt et al., 2017). Similarly, Harach et al reported that amyloid precursor protein (APP), presenilin 1 (PSEN1) transgenic mice had a higher composition of *Bacteroidetes* and *Trichomonas* and less *Proteobacteria* and *Actinomycetes* than wild type age matched mice (Harach et al., 2017)

Due to limited funding, small numbers of samples were sequenced in this project. It is crucial to add more samples to each group to increase sample size and produce statistically significant results. In addition, it is advisable to supplement the fecal samples collected from our experimental groups with CSF to add biomarker detection to correlate with bacterial taxa discovered.

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