Investigating the preventive impact of Indigenous Probiotics along with Quercetin on $AlCl_3$-induced Alzheimer's Disease rat model

By

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List of Abbreviations

(CTF)-“	C-terminal fragment alpha
8-OHdG	8-Hydroxy-deoxyguanosine
AChe	Acetylcholinesterase
ACTH	Adrenocorticotropic hormone
AD	Alzheimer's disease
amyloid-β	Amyloid-beta
ANS	Autonomic nervous system
APP	Amyloid precursor protein
BACE-1	beta-secretase-1
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
cdk-5	cyclin-dependent kinase-5
cdk-5	cyclin-dependent kinase-5
CFU	Colony forming unit
ChAT	Choline acetyltransferase
CNS	Central nervous system
CoQ	Coenzyme Q
CREB	cAMP-response element binding protein
CRF	Corticotropin-releasing hormone
DC	Diseased control group
DPN	Diabetic peripheral neuropathy

ENS	Enteric nervous system
EPM	Elevated plus maze
ETC	electron transport chain
FA	Ferulic acid
FAO	Food and Agriculture Organization of the United Nations.
GABA	Gamma-aminobutyric acid
GBA	Gut brain axis
GI	Gastrointestinal
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSK-3b	Glycogen synthase kinase-3b
GSK-3b	Glycogen synthase kinase-3b
H&E	Hematoxylin and eosin
HPA	Hypothalamic-pituitary-adrenal axis
LAB	Lactic acid bacteria
MAPK	Mitogen-activated protein kinase
MAPT	Microtubule-associated protein tau
MGBA	Microbiota gut brain axis
MRS	De Man, Rogosa and Sharpe
MWM	Morris water maze
NC	Negative control group

NDs	Neurodegenerative diseases
NFT	neurofibrillary tangles
NGF	Nerve growth factor
NOR	Novel object recognition
Nrf2	Nuclear factor erythroid 2-related factor 2
NTS	Nucleus tractus solitarius
PC	Positive control group
PKC	Protein kinase C
ROS	Reactive oxygen species
sAPPα	Soluble ectodomain of amyloid precursor protein
SCFAs	Short chain fatty acid
SOD	superoxide dimutase
TP	Probiotic treatment group
TPQ	Probiotic and quercetin treatment group
TQ	Quercetin treatment group
Trkb	Tyrosine protein kinase
VN	Vagus nerve
WHO	World Health Organization

ABSTRACT

Alzheimer's disease (AD), the most common progressive neurodegenerative illness in older individuals, is characterized by declining cognitive ability. Cognitive decline and memory loss are two of the first pathological signs of Alzheimer's disease by many factors including oxidative brain damage. Several studies have reported improvement in memory and cognition impairment by the consumption of high doses of *Limosilactobacillus* strains and phytochemicals like Quercetin. Through promoting the growth of the HPA axis and the manufacture of neuromodulators such antioxidant enzymes, GABA, SCFAs, serotonin, and BDNF, the gut microbiota influences the gut-brain axis. The current study's objective is to investigate the preventive effect of potential probiotic strains *Limosilactobacillus fermentum* Y55, FM6 and *Lacticaseibacillus rhamnosus* Y59(1.5x109 CFU) and Quercetin (25mg/kg) in combination on AlCl3- induced Alzheimer rat models. Rats were assigned to five distinct groups at random (n = 6). Using a variety of behavioral tests, including the Elevated Plus Maze (EPM), New Object Recognition (NOR), Y-maze, and Morris Water Maze, researchers investigated the impact of this combination on anxiety and memory (MWM) to determine whether recovery plays a part in preventing Al's neurotoxic effects. Male rats were exposed to defined probiotic strains and in combination with quercetin for 75 days before inducing Alzheimer to check the preventive effect of probiotics and polyphenol combination. Then male rats were exposed to AlCl3 toxicity to induce Alzheimer for 75 days. After completion of treatment their learning and memory was tested using Elevated Plus Maze, Novel object recognition test, y maze test and Morris water test paradigms and collated with the controls. To assess the anxiety, an elevate plus maze test was

performed. The animals from treated and their controls were euthanized and dissected after the behavioral tests and the brains were removed to extract hippocampus for further histological analysis. The results unfolded those rats treated with probiotic and polyphenol combination had exhibited significantly improved working, reference memory, recognition and spatial memory with reduced anxious behaviors. Histologically, this combination has improved the normal architecture of the brain hippocampus in TPQ group. H & E staining showed that TPQ group had normal morphological features with proper round and intact cell bodies as compared to DC group. To cap it all, this study demonstrates that animals exposed *Limosilactobacillus fermentum* Y55, FM6 and *Lacticaseibacillus rhamnosus* Y59 and quercetin together show marked improvement in their cognitive functions and memory and improves the morphology of the main targeted parts of brain that is hippocampus.

CHAPTER 1

INTRODUCTION

Alzheimer's disease (AD), the most prevalent cause of dementia, is distinguished by two distinct types of lesions: amyloid β- (Aβ) plaques and intracellular neurofibrillary tangles (NFT) (Jellinger & Attems, 2007). AD pathology includes increased oxidative stress, synapse loss, localized cell death, and brain atrophy(Tönnies & Trushina, 2017) . There are several fiercely debated theories that attempt to explain the fundamental factor that triggers the onset of AD brain illness. In the past, work has been done to link several aberrations together under a single, basic pathogenic mechanism (Workgroup & Khachaturian, 2017). The optimal methods for treating and preventing AD are still unknown since the science lacks consensus on its etiology and pathology after decades of intensive research(Amtul, 2016).

Approximately 60 to 80% of all dementia cases are caused by Alzheimer's disease (AD), which mostly affects people 65 years or older (Association, 2017). 4.6 million new cases of AD are thought to be recorded annually, affecting around 35.6 million individuals worldwide. Age is a factor in the prevalence of AD; starting at 60 years old, the rate doubles every five years (Duthey, 2013). Amyloid-(Aβ) aggregate buildup and tau protein hyperphosphorylation, which result in neurofibrillary tangles (NFTs) and synaptic dysfunction, are commonly linked to the pathophysiology of AD (H.-C. Huang & Jiang, 2009).

Oxidative damage and inflammation are two important systemic factors that make neurodegeneration worse. Both of these are made worse by the normal bodily decline that comes with getting older. Reactive oxidative species (ROS) are formed when 0.4–4% of the electrons moving through the electron transport chain (ETC) escape and combine with an oxygen molecule to generate superoxide radicals, which are the principal source of ROS in the body (Ježek &

Hlavatá, 2005). Ordinarily, the cells' anti-oxidant defense mechanisms convert these free radicals into harmless species, but as people age, their cellular defenses gradually deteriorate, accumulating genetic, cellular, and membrane damage until death of cell occurs (Mehlhorn, 2001).

Over time, ROS builds up in neurons, which causes microglia to become active, cytokines to be released, and neuroinflammation to happen. Oxidative damage and inflammation can cause inflamm-aging, which is a low-level systemic proinflammatory state with high cytokines and inflammatory markers that doesn't have a clear cause. Aging via inflammation is the aggregate name for this vicious cycle." (Almadori & Kalaskar, 2017) To keep oxidative homeostasis, the body needs complicated regulatory systems that can set off self-limiting effector reactions in response to different kinds of oxidative stress. It is well known that Nrf2 (a redoxsensitive transcription factor) plays a key role in controlling antioxidant processes (Zhang et al., 2010). The multitude of antioxidant, cytoprotective, and immunomodulatory enzymes that this gene set encodes help regulate the cellular response to various stresses (Calabrese et al., 2012).

The gut brain axis (GBA) is a communication channel that the central nervous system (CNS) and the enteric nervous system (ENS) maintain(Samtiya et al., 2022). The GBA connects the peripheral intestinal functions to the brain's emotional and cognitive processes. The gut microbiome is a crucial GBA variable (Ghezzi et al., 2022) . The gut microbiota, the dynamic endocrine organ, contains trillions of symbiotic bacteria that have an impact on the host's health. (Sirisinha, 2016). Lifestylerelated changes in the bacterial community's interindividual diversity affect human biochemistry and disease resistance, including the susceptibility to brain illnesses, as well as a number of other alterations in human physiology (Valle Gottlieb et al., 2018). Through a variety of endocrine, neurological, and biochemical processes, the gut microbiota is able to communicate

with the brain in a communication system that is known as the gut-brain axis. This system is bidirectional (Arneth, 2018). The gut microbiota is in a very delicate equilibrium when it is healthy. Disorders or diseases can result from changes that disrupt this microecological balance owing to internal or external sources (Sommer et al., 2017). Bifidobacterium, Eubacterium rectale, and Dialister are a few examples of the beneficial bacterial taxa that are less common in patients with AD's gut microbiota imbalance, while Escherichia coli, Bacteroides, and Ruminococcus are examples of the potentially pathogenic microbes that are more prevalent (Wu et al., 2021) .It has been shown that the variety of the gut microbiota declines with advancing age, which may play an important part in the development of neurodegenerative diseases (Dinan & Cryan, 2017).

Research suggests that the gut microbiota, especially when it is dysbiotic, may influence the development of neurological disorders and even cause them (Kandpal et al., 2022). It is significant to remember that environmental challenges, especially oxidative stress, modify the relative balance of the microbiota population and, in turn, its metabolic and genomic expression. This results in extensive physiological alterations in metabolism and endocrine signaling in the human host (Fan & Pedersen, 2021).

Because of its complex aetiology, AD does not respond well to a single therapeutic approach. A mixture of anti-inflammatory, cholinergic, and antioxidant drugs is used for the symptomatic therapy of AD (Doroszkiewicz & Mroczko, 2022) . Currently donepezil, rivastigmine, and other acetylcholinesterases are used to halt AD(Marucci et al., 2021). They cure symptoms but do not stop the spread of the disease. Also, they are linked to adverse effects that range in intensity from mild, like vomiting and weight loss, to severe, including bradycardia and sleeplessness (Ruangritchankul et al., 2021).

Therefore, modifying the variety of gut microbiota and oxidative stress may be a viable therapeutic avenue, as evidenced by the role of bacteria in gut health and brain function. A comparatively novel and alternative approach that is currently being developed is the combination of probiotics and polyphenols. According to the definition of WHO, “probiotics are live micro-organisms when administrated inadequate amount confer health benefits(Olabode & Akinnate, 2018) . Lactic acid bacteria are usually probiotics and have been declared as safe by FAO and (Zielińska & Kołożyn-Krajewska, 2018). Probiotics have been shown to reverse the dysbiosis of the gut microbiota, slow the onset of AD, especially in cases where inflammation and oxidative stress are present, and lessen cognitive loss (Wong et al., 2018).

Probiotic administration has been demonstrated to lower several oxidative stress and inflammatory markers in AD patients, demonstrating a gut-brain connection throughout the course of the illness (Den et al., 2020). Probiotics are also known as "the cell factory" because of the various bioactive metabolites they generate, and they are essential for preserving human health (Mounir et al., 2022). Lactic acid, SCFA and acetic acid, antibiotic substances, and neurotransmitters are among those metabolites that probiotics create(You et al., 2022). Probiotics create short-chain fatty acids, which prevent amyloid from aggregating and regulate a number of signaling pathways(D’Argenio & Sarnataro, 2021). As a result, there is a decrease in neuro-inflammation, the production of amyloid plaques, and a positive regulation of repressed receptors (Meraz-Ríos et al., 2013).

Flavonoids are phenolic chemicals found in many vascular plants that are isolated from them. The antioxidant effects of flavonoids come from their ability to scavenge free radicals and limit the formation of free radicals. Quercetin is an antioxidant flavonoid found in apples and onions that also raises glutathione levels and modifies the activity of enzymes that combat free radicals (Xu et al., 2019). There are numerous therapeutic plants that contain the flavonoid molecule quercetin

(3, 5, 7, 3′, 4′-pentahydroxyflavone). Apparently acting as an antioxidant, quercetin scavenges reactive oxygen species (ROS) (Mehrbod et al., 2020) . Moreover, it has antiviral, anti-inflammatory, and anticancer effects. Several studies have shown quercetin to have anti-amyloidogenic and neuroprotective benefits against neurological disorders (Aghababaei & Hadidi, 2023).

In Pakistan, numerous studies are being conducted with an emphasis on the industrial and health advantages of polyphenols and probiotics. The current study illustrates how many GBA signalling elements may be simultaneously altered by probiotics and a polyphenol plant extract to prevent AD from initiating and postpone its progression, potentially through antioxidant pathways. Although though both the probiotic formulation and quercetin can independently trigger effects on some of the risk variables of AD, the formulation is the only one that can consistently reduce all the studied risk factors, making the combinatorial therapy more effective than any of its ingredients.

OBJECTIVES

To investigate the potential of *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6, and Quercetin in delaying the progression of Alzheimer's disease in rats induced with AlCl3, focusing on assessing changes in anxiety, learning-related cognitive deficits, and memory impairment through behavioral tests.

CHAPTER 2

LITERATURE REVIEW

2.1 Alzheimer's Disease

Alzheimer's disease is a progressive age-related neurological condition that German neuropathologist Alois Alzheimer originally named Dementia Praecox in 1907. At the 37th Congress of German Psychiatrists, Alzheimer presented his findings and noted that "one or more fibres stand out in the centre of an almost normal cell due to their unique thickness and peculiar impregnability." There are numerous little foci in the topmost layers. They are determined by the storage of specific substances in the cerebral cortex. Overall, we are dealing with a strange disease process" (Hippius & Neundörfer, 2003).

The hallmarks of AD are now understood to be the impenetrable fibres and the millary foci, which were subsequently identified as neurofibrillary tangles and amyloid-based neuritic plaques, respectively. As there was little reaction to Alzheimer's discovery, senility and dementia were still seen as natural effects of ageing. Dr. Emil Kraepelin first referred to AD as a particularly severe type of senile dementia in 1910 (Yang et al., 2016). In the US, AD is the sixth leading cause of death. It is believed that the incidence of AD ranges from 1–3%, while its prevalence ranges from 10–30% of the population over the age of 65. This is due to the fact that AD typically lasts for 10 years ("2023 Alzheimer's Disease Facts and Figures," 2023).

The most prevalent type of dementia among adults is Alzheimer's disease. It is a commonly identified neurodegenerative disease that deteriorates with time (Kumar et al., 2023). The disease has a very high death rate and is tightly linked to both hereditary predisposition and advancing age. The prevalent disease AD is a major contributor to dementia, which begins with an

unchangeable impairment in episodic memory and develops to a more general reduction in overall cognitive function in the elderly. Dementia begins with Alzheimer's disease (AD) symptoms and ends with dementia symptoms (Grand et al., 2011)Disorientation and a slow decline in memory and intelligence are hallmarks of AD (Tarawneh & Holtzman, 2012).

Clinically, the illness shows up as a gradual decline in behaviour and cognition. About 50 million people worldwide suffer from dementia, the most common type of the illness(Cohen, 2013) . By 2050, 152 million cases are expected to occur globallys. Primary carers for AD patients have negative emotional and physical effects as well. According to a study, 37% of nursing personnel who care for AD patients self-reported high emotional strain and 32% self-reported high physical stress (Andreakou et al., 2016).

2.2. Pathophysiology of AD

Alzheimer's disease begins with the accumulation of two proteins in the brain. These proteins, called tau and A-beta, respectively, result in intracellular neurofibrillary tangles and exterior neuritic plaques. Following this are inflammatory responses, neural dysfunction, and neuronal death. β-amyloid-converting enzyme and γsecretase, rather than α-secretase and γ-secretase, break the amyloid precursor protein (APP), releasing A peptides that cause plaques and tangles to accumulate (MacLeod et al., 2015). Amyloid plaque buildup, hyperphosphorylation of tau protein, mitochondrial dysfunction, neuroinflammation, and oxidative stress are all signs of AD pathogenesis (Rajmohan & Reddy, 2017).

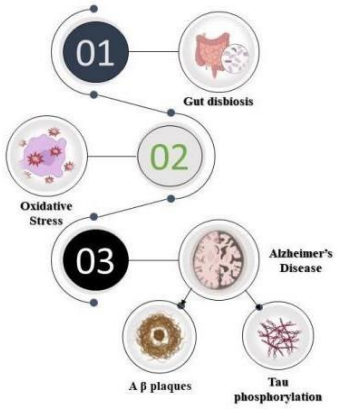


Figure 1: Pathophysiology of Alzheimer's disease

2.3 Hypothesis regarding AD

Notably, there are a number of well-established theories regarding Alzheimer's disease (AD), including the oxidative hypothesis, the tau protein hypophosphorylation theory, and the amyloid-beta cascade hypothesis, which may help identify the pathogenic mechanisms underlying the illness. Oxidative stress is a component that is common to the aetiology of neurodegenerative illnesses, including multiple sclerosis, AD, Parkinson's disease, and Huntington's disease (Singh et al., 2019).

2.3.1 Amyloid beta Cascade Hypotheses:

The theory's initial investigation was carried out in 1991 (Karran et al., 2011). Paul Blocq and George Mannesco originally postulated A-beta plaques in 1892 after observing "circular buildup in the brains of aged patients." Following nearly a century of investigation, Glenner extracted "beta-amyloid" from Alzheimer patients' meningeal arteries and made a partial discovery of the peptide sequence ((Imbimbo & Speroni, 2005). The first non-amyloidogenic pathogenic pathway involves the role of α- and γ-secretases in normal nerve cell synthesis of neurotrophic and neuroprotective substances(Agostinho et al., 2015). In the case of anomalous circumstances, this approach is taken. The soluble ectodomain of APP- (sAPPα), the C-terminal fragment (CTF)-α, and several smaller fragments make up these products.

The first pathogenic pathway, which is not amyloidogenic, is used to create these compounds. The second technique is known as the "amyloidogenic pathological route," in which APP is broken down by γ-secretase into CTF-β and then into various lengths of Aβ peptides, including Aβ42. Aβ42 has a higher degree of neurotoxicity and a larger propensity for plaque formation and aggregation than (Irie et al., 2005). Additional effects of Aβ include a significant reduction in

spatial working memory, an increase in cholinergic dysfunction, a decrease in ChAT and AChE activity, and an increase in AChE activity in the hippocampus, amygdala, and prefrontal cortex. Additionally, certain areas of the mouse brain show noticeably more apoptosis. Furthermore, A significantly reduced mitochondrial integrity, bioenergetics, and function in every region of the rat brain (Bernardo et al., 2016).

2.3.2 Tau Hypothesis

Tau is a microtubule-associated protein that is produced only when a certain kind of alternative splicing takes place in the MAPT gene. For the first time, Claude Wischik showed that tau protein might be the main cause of dementia when he separated tau from plaques discovered in the brains of Alzheimer's patients in 1988 (Wischik et al., 2014). The brain's neuronal axons contain microtubules and tau (Terwel et al., 2002). Tau protein interacts with tubulin through its isoforms and phosphorylates its own protein to stabilise microtubule assembly. The tau protein family consists of six isoforms with amino acid ranges of 352-441. While the CNS isoforms with the shortest lengths, R1, R3, and R4, include only three repeats and no inserts (352 amino acids total), the longest-lengthed isoforms, R1, R2, R3, and R4, contain a total of 441 amino acids split among four repeats and two inserts. According to (Planel et al., 2002), all six tau isoforms are present in the paired helical filaments that are indicative of Alzheimer's disease, and many of them are hyperphosphorylated. GSK-3b, or glycogen synthase kinase, and cyclin-dependent kinase-5 (cdk-5) are two of the several kinases linked to the pathogenic process of tau hyperphosphorylation. Several tau phosphorylation sites are among the ones they can target (Churcher, 2006). The hyperphosphorylation of tau protein causes the cytoskeleton to become unstable, which leads to the degeneration of nerve cells. In this statement, it is important to remember that tau is essential for the stability of cytoskeletal microtubules (Gong & Iqbal, 2008).

2.3.3 Significance of Oxidative Stress in AD

Oxidative stress is the result of unbalanced antioxidant and free radical levels. Oxidative stress is also another precursor to AD (W.-J. Huang et al., 2016). According to (Apostolova et al., 2011), reactive oxygen species, also known as free radicals, are thought to play a role in oxidative cell damage and cell death. Of these, 95–98% are produced as byproducts of the electron transport chain (ETC) in the mitochondria. The brain is an organ that requires a high oxygen content to operate properly, making it susceptible to the effects of reactive oxygen species (ROS). A large amount of oxidation-sensitive polyunsaturated fatty acids are present in the brain, along with low concentrations of enzymes and other antioxidant molecules. Iron is a powerful ROS catalyst and is present in considerable amounts in it as well (H. Palacios et al., 2011). Usually, the reactive molecules that comprise ROS include oxygen. Compounds that easily generate free radicals, with reactivity ranging from low to high, or free radicals themselves are the unstable, transitory, and highly reactive ROS (Lushchak, 2014).

According to Abramov et al. (2020), an excess of free radicals may be responsible for the accumulation of tau and beta-amyloid (Aβ) proteins, which are two of the main indicators of Alzheimer's disease (AD)(Atlante et al., 2021). Free radicals are essential for synaptic plasticity at physiologically appropriate levels, which impacts memory and learning. However, synaptic plasticity and memory are hampered in neurodegenerative diseases such as AD when oxidative stress is increased (Agostinho et al., 2010). Oxidative stress has been the subject of much research as a therapeutic target for the management of learning and memory impairments in AD. In the nucleus and mitochondria of Alzheimer's patients and animal models, there is an increased level of 8-Hydroxy-deoxyguanosine (8-OHdG), a biomarker of DNA oxidative damage (Buccellato et al., 2021).

When complex I and coenzyme Q (CoQ) accumulate electrons, it is because ETC is blocked in Alzheimer's disease. The superoxide radical (O2) and nitric oxide (O-) can be produced by directly exposing the accumulated electrons to molecular oxygen, and the O2 radical can then combine to form the peroxynitrate radical (OONO-). When transition metals are present, the H2O2 transforms into the dangerous hydroxyl (OH) radical. Thus, AD is associated with ETC dysfunction and the production of free radicals. One more characteristic of AD is its lack of antioxidant capacity. Low glutathione levels are accompanied by decreased activity of the enzymes Cu/Zn SOD (superoxide dismutase) and GSH. Antioxidants cannot therefore prevent free radicals from damaging cells (Wojtunik-Kulesza et al., 2016).

2.4 Gut-brain Axis

It was theorized that "nervous weakness" may result in defective digestion, or on the other hand, that impaired digestion may permit accumulation of toxins from the intestines, including bacteria, that disrupt the action of the nervous system. The concept of connectivity between the stomach and the brain dates back as far as the eighteenth century. At the time, physicians reported on such illnesses as "neurasthenia gastrica" and "autointoxication" (M. Sun et al., 2020).

The gut-brain axis, which interacts in both healthy and sick states, is an anatomical connection between the gut and the central nervous system (CNS) (A. Kohler et al., 2016). Understanding the crosstalk between the gut and the brain has revealed a complicated communication system that ensures the proper maintenance of gastrointestinal homeostasis. Moreover, several effects on mood, motivation, and higher cognitive abilities are anticipated from this system. "Gut-brain axis" (GBA) is a phrase that describes the intricate nature of these relationships (Sánchez Aragón, 2022). Its job is to keep an eye on and integrate gut processes while also connecting the brain's affective

and cognitive regions to peripheral intestinal processes and mechanisms include intestinal permeability, entero-endocrine signaling, enteric reflex, and immune activation.

The mechanisms underlying GBA communications include the involvement of neuro-immunoendocrine mediators (Swer et al., 2023) . It has been speculated for a long time that the gut may be home to as many as 1014 bacterial cells, which would explain for the reputed 10:1 ratio of bacterial cells to human cells that is frequently mentioned in the literature. Yet, according to recent studies, there are about 3.8× 1013 bacterial cells overall in the gastrointestinal (GI) tract, making the above ratio 3:1 (Naidu et al., 1999). When a newborn is exposed to a variety of environmental stimuli, the gut microbiota grows in size and diversity. By the time an infant is one to two years old, an adult-like microbiome is visible. Because of its dynamic nature, the microbiome of the gut can be affected by a wide range of factors, including the method of birth delivery, the transfer of microbes from the mother to the child, genetics, food, infection, drugs (such as antibiotics), age, and stress (Tochitani, 2021). These factors have the potential to not only temporarily but also permanently alter the composition of the gut. Other phyla, such as Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia, are found among the microbial diversity of gut, although in substantially lesser levels(Ghosh & Pramanik, 2021). Firmicutes and Bacteroidetes are the two bacterial phylotypes that predominate in the gut microbiome. Despite the fact that each person's gut microbiota is unique, people have been classified into three "enterotypes", each of which is dominated by a different species of bacteria: Prevotella, Ruminococcus, and Bacteroides (Colella et al., 2023).

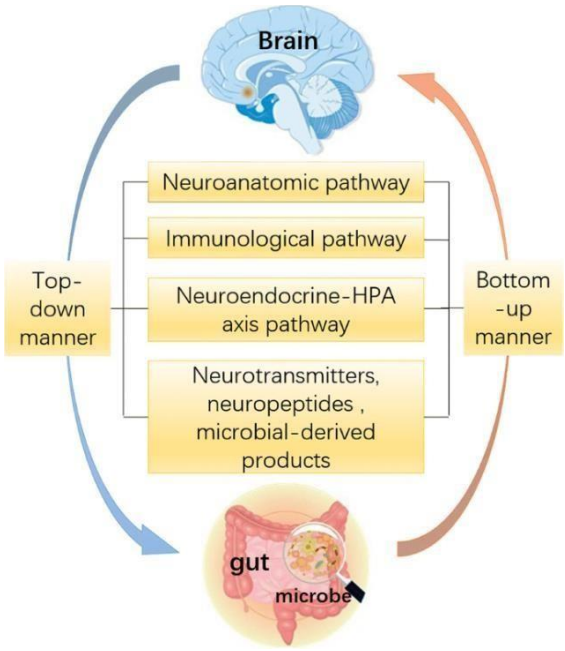


Figure 2: Bi-directional pathway of Gut Microbiota-Brain axis (Zhao et al., 2018)

2.5 Bi-directional communication routes of Gut brain axis

Because the gut microbiota affects the central nervous system (a bottoms-up approach) and the central nervous system (CNS) affects the makeup of the gut microbiota, the gut-brain axis is thought to be bidirectional. Stated differently, the gut microbiota influences the central nervous system (CNS) in a top-down manner. Through the autonomic nerve system and the hypothalamic-pituitary-adrenal axis, the brain can regulate the function of the gastrointestinal tract. For example, when under stress, the brain releases norepinephrine, which has been demonstrated to promote the growth of intestinal infections (Mittal et al., 2017). On the other hand, the gut uses a variety of metabolites and products formed from the microbiota, as well as gut hormones and neuroactive substances that pass through the enteric nervous system, to affect the functioning of the central nervous system (Yarandi et al., 2016).

2.5.1 Vagus nerve

There are 80% sensory afferent fibres and 20% motor efferent fibres in the vagus nerve (VN), commonly known as cranial nerve X (Yuan & Silberstein, 2016). The 10th cranial nerve, the vagus nerve, connects the brain to the gut's muscular and mucosal layers. It regulates satiety, GI secretion, and the gut's peristalsis, among other functions (Altaf & Sood, 2008). It is the parasympathetic nerve system's main constituent. It connects the brain and microbiota through the vagal circuit. Afferent vagal neurons are stimulated by the microbiota and other gut chemicals, which results in the firing of action potentials and the release of glutamate, a neurotransmitter that is largely excitatory, in the nucleus tractus solitarius (NTS).

2.5.2 HPA axis

The neuroendocrine system's HPA axis regulates numerous biological functions and responds to both physical and psychological inputs. Studies indicate a direct correlation between the gut microbiota and the HPA axis. The action of the HPA axis modifies the composition of the gut microbiota and increases gut permeability, which facilitates the entry of bacterial compounds into the bloodstream and results in persistent low-grade inflammation (Rea et al., 2016). Cortisol levels decreased in healthy human volunteers who ingested the B. longum1714 strain. This hippocampal-dependent translational psychobiotic concurrently improved visuospatial memory function. In an animal model of partial limit stress, there was a rise in plasma adrenocorticotropic hormone (ACTH), pro-inflammatory cytokines, corticosterone, hypothalamic corticotropin-releasing hormone (CRF), and intestinal permeability. Combining several probiotics reduced intestinal permeability, inflammation, and the HPA axis' stress response (Ait-Belgnaoui et al., 2012). Bacterial translocation has been linked to stress-related neuropsychiatric diseases, including depression. Potential probiotic Limosilactobacillus farciminis may control barrier leakiness caused

by HPA response associated to stress (Anand et al., 2023). In ageing mice fed high-fat diets, a human-derived microbiota cocktail containing five different strains of Enterococcus and Limosilactobacillus, respectively, decreased gut dysbiosis, leaky gut, and cognitive decline .

2.5.3 Neurotransmitters and Neuromodulators

Metabolites and products produced by microbes, which primarily impact host tissues or cells via interactions with receptors, are significant contributors to the MGBA. As a consequence of the breakdown of carbohydrates by microbes, short-chain fatty acids (SCFAs) have been shown to promote learning and memory development, glucose homeostasis, lymphocyte function, and mucosal serotonin release via preserving brain integrity (Mirzaei et al., 2021)

Neuroactive molecules, a different class of microbiota-related substances, also influence the MGBA, most likely through the ENS. It has been demonstrated that gut bacteria, if not directly synthesised, alter neuroactive substances that affect the central nervous system, such as dopamine, noradrenaline, acetylcholine, melatonin, histamine, and GABA ((Wall et al., 2014). In goats, consuming Macleaya cordata extract and Limosilactobacillus plantarum also reduced intestinal oxidative stress and preserved the intestinal mucosal barrier . Thus, by promoting antioxidant enzymes, raising the synthesis of antioxidant metabolites, and modifying antioxidant signalling pathways and gut microbiota, consuming probiotic bacteria may serve as a biological barrier to the generation of reactive oxygen species (ROS) (Wang et al., 2017). Additionally, polyphenols contribute to the secretion of antioxidant enzymes. In a previous work, the scientists created an AD model using streptozocin. Next, in a rat model of diabetic peripheral neuropathy (DPN), they used quercetin to lessen the degree of damage to the myelin and axons. Furthermore, they enhanced the diversity of gut flora in every group and decreased the amount of ROS generation (Xie et al., 2020).

2.6 Alzheimer's and Gut-brain axis

Alzheimer's is a side effect of peripheral inflammation in addition to limited brain inflammation (Xie et al., 2020). The fact that gut dysbiosis causes inflammation that worsens with age, disrupts the BBB, activates the immune system, and then causes neurodegeneration is evidence for this. On the other hand, a healthy, well-balanced stomach helps to lessen the negative consequences of ROS (Tan et al., 2021). Similarly, the 5xFAD mouse model which mimics Alzheimer's disease demonstrated shifts in the population of microbiota towards proinflammatory species in conjunction with changes in amino acid catabolism. On the other hand, therapy with antibiotics was able to reverse the effects, suggesting a probable link between the severity of the disease and an altered gut population (Langdon et al., 2016).

The 16s ribosomal RNA MiSeq sequencing method was utilised by a Chinese cohort to ascertain the alterations in the gut microbial colonies of individuals with AD. The study found that three significant phyla had radically altered ratios in the gut microbiota of AD patients. Compared to proteobacteria, which exhibited a significantly higher relative abundance ($p = 0.024$), firmicutes had a much lower relative abundance ($p = 0.008$). Additionally, bacterioidetes were less prevalent in AD patients. Firmicutes are significant SCFA-producing bacteria. SCFAs are essential for preserving the permeability of the mucosal barrier and protecting the BBB. BBB deterioration and leaky gut are brought on by a reduction in the quantity of circulating SCFAs. Proteobacteria are proinflammatory microbes that cause the accumulation of LPS by releasing cytokines. In AD individuals, ruminococcaceae and clostridiaceae were less prevalent. Their decreased abundance has been linked to insulin resistance. Insulin resistance is one of the main risk factors for AD (Carranza-Naval et al., 2021). Based on this findings, it may be possible to reduce the onset and

progression of disease by changing the diversity and composition of the microbiome through a number of different methods.

2.7 Conventional treatment for delaying the progression of Alzheimer's disease

The pathogenesis of AD is a complex phenomenon involving multiple pathways, making it extremely difficult to identify a single therapeutic approach. Antioxidant, cholinergic, and anti-inflammatory drugs are used to treat AD symptoms. Certain drugs provide unpleasant side effects and don't stop the progression of neurodegeneration or sickness (Sharman et al., 2019). Blocking acetylcholinesterase (ACE), which recycles synaptic acetylcholine in grey matter, is the most recommended treatment for AD. An initial characteristic of AD is a decrease in cholinergic neuron density. When acetylcholinesterase is suppressed, acetylcholine activity is increased (Alam & Sharma, 2019). Among the more well-known ACE inhibitors include galantamine, rivastigmine, and donepezil. Many side effects, including heart block, bradycardia, insomnia, dizziness, allergic dermatitis, cramping in the muscles, and appetite loss followed by weight loss, are associated with these oral drugs. There might be more detrimental effects; this is by no means an exhaustive list (Sahoo et al., 2018). Researching novel treatments that impede the progression of disease and have fewer side effects is therefore essential.

Alzheimer's disease (AD) presents a formidable challenge in healthcare due to its progressive nature and the absence of a definitive cure. Current treatment options predominantly aim to manage symptoms and slow the disease's advancement. Among the most commonly prescribed medications are cholinesterase inhibitors, including donepezil, rivastigmine, and galantamine, which work by elevating acetylcholine levels in the brain, thereby improving cognitive function in some patients. However, their long-term efficacy is uncertain, and adverse effects such as gastrointestinal disturbances may limit their tolerability. Memantine, another medication used in

AD treatment, targets glutamate regulation to protect brain cells from damage. While it may offer some benefit, especially in later disease stages, its impact on cognitive function is moderate and varies among individuals. Combination therapy involving both cholinesterase inhibitors and memantine has been explored, but evidence supporting its superiority over monotherapy remains inconclusive. Managing vascular risk factors such as high blood pressure, cholesterol, and diabetes is also crucial, as AD is closely linked to vascular health. Lifestyle interventions, including regular physical activity and a balanced diet, may offer some protective benefits against AD progression, although further research is needed to confirm their efficacy over the long term.

Despite these treatment options, conventional therapies have limitations. While they may provide temporary relief from symptoms and potentially slow disease progression in some individuals, they do not halt or reverse the underlying neurodegenerative processes of AD. Additionally, the effectiveness of medications varies from person to person, and side effects can hinder treatment adherence. As such, ongoing research is exploring novel directions to address these limitations. Efforts are underway to develop disease-modifying drugs targeting the underlying pathology of AD, such as amyloid plaques and tau tangles. While initial clinical trials have yielded mixed results, several promising candidates are in development. Personalized medicine approaches, tailoring treatments to individual patient characteristics, show potential for optimizing efficacy and minimizing side effects. Furthermore, combining conventional therapies with innovative interventions like neuromodulation, dietary supplements, and stem cell therapy is being investigated to enhance treatment effectiveness in combating AD progression.

2.8 Alternative therapy for AD

Recently, research has examined the role of the microbiome in relation to neurodegenerative diseases (Zhu et al., 2021). Furthermore, it is now shown that the axis connecting the gut and the

brain plays a significant role in the onset of neurodegenerative diseases. Therefore, probiotics and polyphenols, which are crucial for maintaining a healthy microbiome, are among the special chemicals that may be utilized in AD prevention (Serra et al., 2018).

2.8.1 Probiotics

The current definition of probiotics is derived from the Food and Agricultural Organization of the World Health Organization and reads as follows: "live bacteria that, when delivered in suitable proportions, impart a health benefit on the host." By emphasizing that bacteria "when taken in suitable proportions as part of food" have beneficial effects, the concept of food can be altered (Joint FAO/WHO Expert Consultation On, 2001). The Italian Ministry of Health (IMH) advises ingesting at least 1x10^9 CFUs of live probiotic microorganisms daily for health benefit.

According to (Sánchez-de-Lara-Sánchez & Sánchez-Pérez, 2022), probiotics are a widely used, affordably priced, and well-tolerated medication. Probiotics have a number of methods of action, yet the exact mechanism by which they work is still not fully understood. These encompass a wide range of processes, such as the production of bacteriocin and short chain fatty acids, nutritional competition, reduction of gut pH, and activation of mucosal barrier function (Y. Sun & O'Riordan, 2013). Probiotics have been demonstrated to enhance cognitive decline and to delay the course of AD, particularly the oxidative stress and inflammatory response.

Probiotics can also assist in restoring the natural balance of intestinal flora (Fooks & Gibson, 2002). Apart from their antimicrobial properties, they also maintain intestinal lining integrity, adjust the body's pH, and promote the synthesis of neurotrophic factor (Peng et al., 2020). The proteins in the brain that are referred to as BDNF help neurons survive and grow. It is therefore necessary for neurological development. Common issues including cognitive disabilities and

memory impairments may arise if these brain components are missing (Balaratnasingam & Janca, 2012).

Probiotics improve CNS functions and the composition of beneficial bacteria in the gut microbiota, which both have positive effects on the central nervous system (CNS). Because probiotics directly affect brain biochemical substances including serotonin, -aminobutyric acid (GABA), and dopamine, they have an excellent prognosis for treating memory loss and mental health issues in addition to brain neurotrophic factor (Autry & Monteggia, 2012). The two probiotic bacterial genera that are most commonly used are Bifidobacterium and Limosilactobacillus. Eating them does not cause inflammation of any type because they do not contain lipopolysaccharides (Piccioni et al., 2021). In AD rats, the effects of probiotics on memory, spatial learning, and other aspects have been investigated. Rats administered probiotics (Bifidum, B. longum, and L. acidophilus) for four weeks showed a substantial increase in lipid profiles, long-term potentiation, paired-pulse facilitation ratios, and spatial learning and memory compared to the AD group(Rezaeiasl et al., 2019).

Probiotics have garnered significant attention for their potential in delaying the progression of Alzheimer's disease (AD) due to their multifaceted mechanisms of action. One key mechanism involves their ability to modulate the gut microbiota composition, thereby influencing the gut-brain axis. Dysbiosis, or imbalance in gut microbiota, has been linked to neuroinflammation and cognitive decline observed in AD. Probiotics, such as Bifidobacterium and Limosilactobacillus, promote the growth of beneficial bacteria while suppressing harmful ones, restoring microbial balance in the gut. This restoration of gut microbiota balance can help alleviate neuroinflammation and reduce oxidative stress, both of which are implicated in the pathogenesis of AD.

Furthermore, probiotics exert direct effects on the central nervous system (CNS) by influencing neurotransmitter production and release. For example, probiotics have been shown to increase serotonin, gamma-aminobutyric acid (GABA), and dopamine levels in the brain, all of which play crucial roles in regulating mood, cognition, and memory. Additionally, probiotics can enhance the synthesis of brain-derived neurotrophic factor (BDNF), a protein vital for neuronal survival, growth, and plasticity. By promoting the production of BDNF, probiotics support neurogenesis and synaptic plasticity, crucial processes for maintaining cognitive function and memory formation. Overall, the combined effects of probiotics on the gut microbiota and CNS function contribute to their potential in delaying the progression of Alzheimer's disease. Through these mechanisms, probiotics offer a promising avenue for AD prevention and management, although further research is needed to fully elucidate their therapeutic potential and optimize treatment strategies.

2.8.1.1 Anti-Alzheimer potential of *Limosilactobacillus fermentum and Lacticaseibacillus rhamnosus*

Increased levels of oxygen radicals within the cell due to oxidative stress can damage macromolecules like proteins and nucleic acids. Enzymes contained within organisms deal with ROS, neutralizing them and preventing the harm they cause. The main defense mechanisms that shield the body from the harmful consequences of oxidative stress include the enzymes SOD, GPx, GR, and non-enzymatic antioxidants (Adwas et al., 2019). The use of biologically derived antioxidants is becoming more and more common as a means of promoting antioxidants (Gulcin, 2020).

Redox-active metals such as cobalt (Co), copper (Cu), and iron (Fe) induce redox reactions, which in turn trigger the generation of reactive oxygen species (ROS) (Jomova et al., 2012). Probiotic

usage may provide a number of extra antioxidant benefits. One strategy is the observation that some lactic acid bacteria have been shown to be able to chelate metal ions, even though the exact mode of action is still unknown (Mirza Alizadeh et al., 2022).

Lactic acid bacteria have the ability to produce antioxidant enzymes on their own in addition to promoting the host's production of them. For example, it has been shown that SOD is present in Limosilactobacillus fermentum. (2002) Kullisaar et al. Studies utilizing mice as models have demonstrated the potential of bacterial SODs in the treatment of Crohn's disease (Bryukhanov et al., 2022). Furthermore, it is known that *Limosilactobacillus fermentum* and *Lacticaseibacillus rhamnosus* activates several pathways, such as Nrf2-Keap1-ARE, PKC, and MAPK, which function as a host's defense mechanism against oxidative stress (Averina et al., 2021). This lactic acid bacterium not only activates the Nrf2 pathway but also Nrf2-related antioxidant enzymes such as SOD, catalase, and heme oxygenase 1 (Pan et al., 2017).

Ferulic acid affects neurons directly when it comes to neurodegeneration. Studies conducted both in vitro and in vivo have demonstrated that it can promote the growth of brain stem cells. In the former instance, corticosterone administration of mice resulted in an increase in the number of neurons in the dentate gyrus, indicating corticosterone's potent ability to stimulate neurogenesis in vivo. More recently, ferulic acid has been a prominent focus for modulating communication between the commensal microbiota and the brain. Using the ferulic acid esterase gene, certain gut flora may synthesize substantial amounts of FA rapidly in addition to food sources(Leonard et al., 2021).

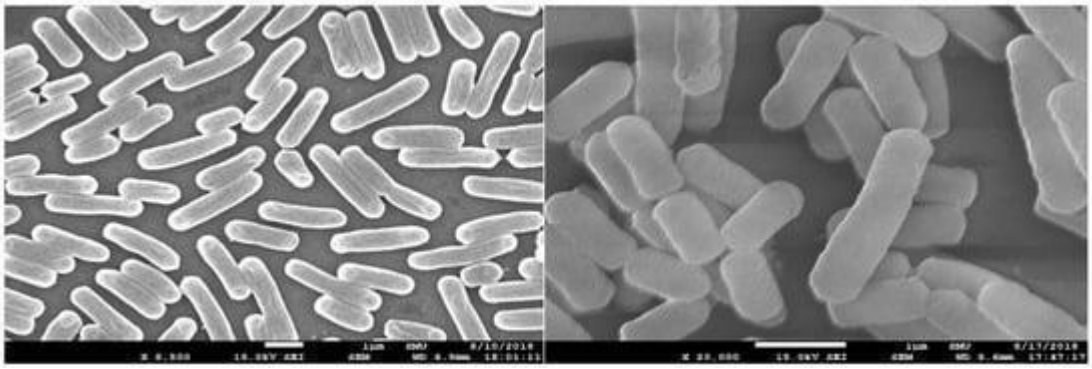


Figure 3: SEM image of Limosilactobacillus fermentum (Ann et al., 2023)

2.8.2 Polyphenols

Secondary plant metabolites, or phytochemicals, are present in a variety of meals and drinks, such as fruit, vegetables, cereals, nuts, cocoa/chocolate, juice, tea, and wine. Generally, dietary phytochemical consumption surpasses 1 g day (Adefegha, 2018). Flavonoids, the most diverse class of phytochemicals, are abundant in higher plants and have remarkable therapeutic potential (Jucá et al., 2020). Flavonoids are further divided into six types based on their chemical composition: flavonols, isoflavonoids, anthocyanidins, flavanols, and flavones (Brodowska, 2017).

Due to their important roles in the pathogenesis of AD, flavonoids' antioxidant and anti-inflammatory qualities have drawn a lot of interest (Ullah et al., 2020). Flavonoids have been found to have the ability to cross the BBB. Therefore, they might be applied to stop neurodegenerative illnesses. However, different flavonoid subclasses have different BBB-crossing capabilities (Phukan et al., 2023). According to (Tian et al., 2018), the efficiency of AD is explained by a reduction in oxidative stress and the toxicity of Aβ.

2.8.2.1 Quercetin

The flavanols are a subgroup of flavonoids, and Quercetin is a flavanol. One of these compounds that people consume the most in their diets is Quercetin, with an average daily consumption of

between 5 and 40 mg (Kelly, 2011). Five hydroxyl groups and three ring configurations make up the chemical makeup of Quercetin. It has the ability to pass BBB, which, in the context of neurodegenerative disease, is critical (Khan et al., 2019) .

Many qualities of Quercetin, such as its anti-inflammatory and antioxidant capacities, are advantageous to human health. This final point is especially important to keep in mind when discussing neurodegenerative diseases because the brain is an organ that is particularly susceptible to oxidative stress. This is because the brain has a high concentration of unsaturated fatty acids, a high rate of oxygen consumption, and a limited capacity for antioxidants (Gilgun-Sherki et al., 2001). It is also possible to obtain it in free form by isolating it from the surfaces of leaf, fruit, or bud extracts. Passiflorae, Compositae, Solanaceae and Rhamnaceae, are plant groups that are high in quercetin (Khan et al., 2019).

Quercetin is present in relatively high percentages in apples, mangoes, red leaf lettuce, onions, tomatoes, asparagus, capers, buck weed, tea, citrus, plums and berries (Kelly, 2011). Due to its ability to operate as a substrate for gut microorganisms to produce SCFAs while remaining undigested until it reaches the colonic section, the flavonoid can also function as a prebiotic (Rawi et al., 2020). In order to check the ability of quercetin on SCFA a study was conducted on rmTBI mice. In faecal samples from rmTBI mice, we saw decreased levels of acetate, propionate, and butyrate. These levels recovered in the animals that had been given quercetin (Balasubramanian et al., 2023).

Quercetin has demonstrated therapeutic potential, enhancing cognitive abilities such as memory and learning in AD. In patients suffering from Alzheimer's , there is evidence that quercetin can improve learning, memory, and other cognitive skills (Sabogal-Guáqueta et al., 2015). In vitro models were used to draw the conclusion that quercetin treatment inhibited the AChE and secretase

enzymes, inhibiting the breakdown of acetylcholine and reducing the synthesis of Aβ, respectively (Khan et al., 2018). In vitro and computer simulation studies have also shown quercetin can decrease beta-secretase-1 (BACE-1) enzyme activity by forming hydrogen bonds. The C-3 OH group has a crucial role in the suppression of BACE-1 (Mezeiova et al., 2019).

Tauopathy frequently starts in the hippocampus, disrupting cognitive functions that depend on it, before spreading to other parts of the brain. It has been proven that quercetin can lower the phosphorylation of tau proteins and can limit the generation of NFTs in mice that have been genetically engineered to imitate age triple transgenic Alzheimer's disease (Navarro-Hortal et al., 2022). The ability of quercetin to scavenge free radicals, chelate metals, and shield neurons from metal toxicity account for the majority of its antioxidant activities It is possible for quercetin to have an effect on enzyme systems, such as the nitric oxide synthase, as well as transcriptional factors, such as Nrf-2, which are responsible for activating genes that code for detoxifying and antioxidant proteins (de Oliveira et al., 2016).

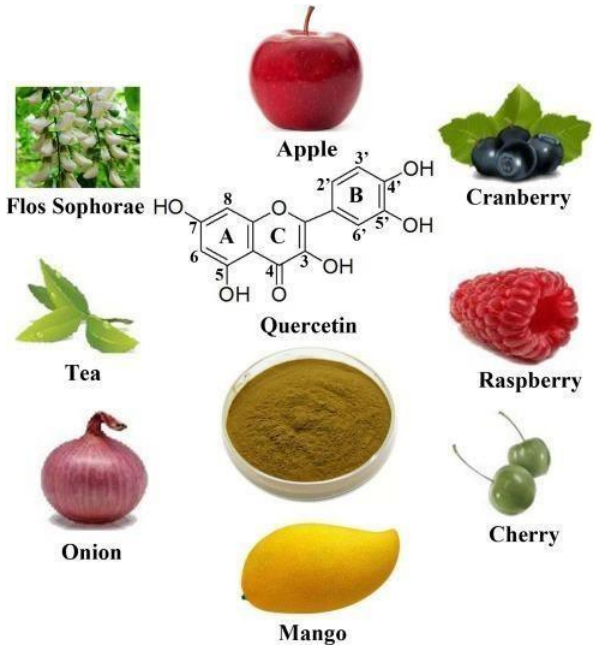


Figure 4 : Sources of Quercetin (Shi et al., 2019)

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

Aluminum chloride hexahydrate (AlCl3.6H2O) was purchased from Scharlau (Product catalogue no. AL0770). MRS Broth and MRS Agar were got from MERK. Chemicals for gram staining like Safranine, Gram's Iodine, Crystal Violet and Decolorizer were bought from Diachem. Quercetin in powder form was purchased from Saitong (Product catalogue # C21PB9883A).

3.1.1 Probiotic strain selection (*Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6)

Probiotic Isolate *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6, were selected that were previously isolated by lab fellow Noor, (2019) and Hafsa Raja, (2022). These strains were further characterized exhibiting antioxidant potential by in-silico analysis done by another lab fellow Ayesha, (2022).

3.2 Morphology assessment of *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6

3.2.1 Gram staining

Single colony was selected from MRS agar plate and dispensed on the droplet of distilled present on the slide to make smear which was then fixed by heat. The smear was then treated for 1 minute with crystal violet and removed with distilled water. It is then treated for 40 seconds with an iodine solution, washed with ethanol for 5 seconds before being stained with safranin for 40 seconds, and finally washed with distilled water. After drying by air, it was examined with immersion oil under 100X objective lens microscope. Then catalase test was conducted. A single

colony of probiotic strain was picked with sterile loop and transferred to dry clean slide. 3% H2O2 was added on the colony and mixed well.

3.3 Animal Trials

Institutional Review Board (IRB) of ASAB, NUST under ethical code "07-2022-ASAB-01/02" approved the present study. Wister rats were purchased and housed in the animal housed in the animal house of Atta ur Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST). Rats were housed in cages with a natural light-dark cycle and a constant temperature of 25°C (12-12 hours). Animals were provided with unlimited access to distilled water and a standard meal of crude protein (30%), crude fat (9%), crude fibre (4%), and moisture (10.4%). Experiments were conducted using male rats (n=30), weighing 50–100 g, and between 2 to 4 weeks old.

3.4 Study Design:

Before initiating the animal trial, probiotics were revitalized from the stocks and underwent gram staining and catalase testing. A dosage of 1.5×10^9 CFUs/mL/day/rat was administered to two groups: the TP group and TPQ group. Probiotic strains—*Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, and *Limosilactobacillus fermentum* FM6—were cultivated for 24 hours in MRS broth, followed by centrifugation for 20 minutes at 5000 rpm at 4°C. The cell pellet underwent washing with PBS multiple times. Subsequently, the probiotics were combined with distilled water, and their turbidity was compared to the 0.5 McFarland standard. The optical density of the dosage was matched with the 0.5 McFarland standard, ranging from 0.081 to 0.1. Fresh dosages were prepared on the day of administration.

A three-month plan was devised to evaluate the impact of *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6, and Quercetin in an

AlCl3-induced Alzheimer's rat model. The study aimed to compare the effects of probiotics and Quercetin administered separately and in combination in the Alzheimer's model. Behavioral tests assessing memory impairment and anxiety were conducted, followed by animal decapitation for histopathological studies.

Sr No.	**Experimental Groups**	Sample Size (n)	First 75 Days Treatment	Additional Treatment (Next 75 Days)
1.	Control group	6	Distilled H2O	Distilled H2O
2.	Diseased group	6	Distilled H2O	120 mg/kg AlCl3
3.	Probiotic Treated group	6	Probiotic 1.5×10^9 CFUs	Probiotic 1.5×10^9 CFUs (1.5 months) +120 mg/kg AlCl3
4.	Quercetin Treated group	6	25mg/kg Quercetin	25mg/kg Quercetin +120 mg/kg AlCl3
5.	Probiotic & Quercetin Treated group	6	Probiotic 1.5×10^9 CFUs + 25mg/kg Quercetin	Probiotic 1.5×10^9 CFUs + 25mg/kg Quercetin + 120 mg/kg AlCl3

3.5 Behavior studies

Behavior tests were done during the light cycle of rats, from 9 a.m. to 5 p.m., so that the rat's circadian rhythms would not cause any changes. Before beginning the behavior experiment, the rats were habituated for 30 minutes in the appropriate behavior room. The temperature was kept at 22 ± 2°C, and the room was sufficiently illuminated. The level of human intervention and disturbance was kept to a minimum. For video recordings of behavior tests, a video camera was

connected to a tripod stand (S. Lee et al., 2012)**.**

3.5.1 Elevated plus maze test (EPM)

The EPM is used all over the world to examine the psychological and neurochemical causes of anxiety as well as to test rodent genotypes and anxiety-modulating drugs. Both conventional anxiety indices and ethologically derived "risk assessment" behavior are tested using the elevated plus-maze. There are many different regions involved, but the hippocampus and amygdala are the primary ones (Reddy & Kulkarni, 1998).

Apparatus

The elevated plus maze had four arms, two of which were closed alleys and two of which were open alleys. The apparatus had 30 × 5 cm-long arms and was composed of an opaque iron alloy. It was raised 75.5 cm off the ground.

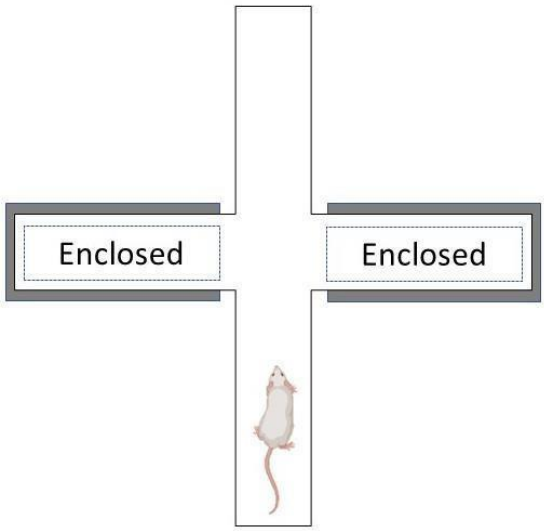


Figure 5 : Elevated plus maze test apparatus.

Procedure

EPM was done in accordance with the established protocol by (Arendash et al., 2004). To start

the experiment, rats were placed in the plus, facing the closed arm, and they were observed for 5 minutes. A spectator sat still 1.5 meters from the maze's center. A video recorder was set to record the behavior of rats in the above-mentioned time. Following parameters were analyzed through the video:

1. Time spent in open arm.
2. Number of entries in open arm.

3.5.2 Y-maze test

A well-established test of spontaneous exploration behavior in rodents is the Y-maze. It has also been referred to as a working memory test, an active or spontaneous working memory test, and a test of spontaneous alternation performance. Animals' natural behavior to explore undiscovered areas serves as its driving force.(Lalonde, n.d.). A mouse with an intact working memory, and consequently an intact prefrontal cortex, will recall the arms that have already been visited and will have a propensity to enter a less often visited arm. During trial period or probe testing, one arm is blocked to test the animal's recollection of the arm that it has never visited before (Kraeuter et al., 2019).

Apparatus

Three light-colored, opaque arms that are 120° apart from one another and each measure 16 cm high, 10 cm broad, and 40 cm long make up the Y-maze. The first arm is the starting arm, the second is the other arm, and the third is the novel arm, which is kept closed during the habituation phase and opened during the trial phase. The starting arm is where the animal is placed (Jung et al., 2020).

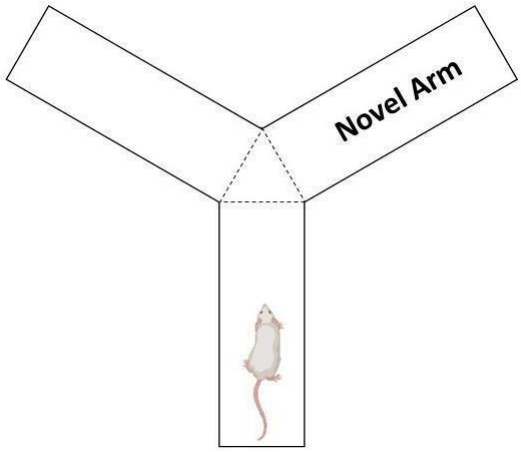


Figure 6 : Y-maze test apparatus

Procedure

Animal was put inside a Y-maze during the habituation period, with the novel arm remaining. closed off and only the start and other arms being open the animal was left free to explore its surroundings for 15 minutes while it was positioned in the start arm of the y-maze, facing the wall of the start arm. The animal was removed from the maze when the habituation period was over and placed back in its cage in order to create a 15-minute intertrial period between the habituation and probe trials (Kraeuter et al., 2019). Animals were then free to roam the maze for around five minutes during the probing phase. A camera positioned above the maze captured the trial. Between habituation, the probing trial, and the following animal session, the maze was thoroughly cleansed and wiped with 70% ethanol to eliminate any odor cues for the animal. The following parameters will be evaluated using recorded videos:

1. Time spent in each arm
2. Spontaneous alternation performance (%)

   % Spontaneous alternation performance = (No. of Spontaneous alternation / total number of entries – 2) × 100

3.6.3 Novel object recognition test (NOR)

This behavior test was created in 1987 by Ennaceur and Delacour (Ennaceur & Delacour, 1987) based on the instinctive need of animals to investigate novelty. It focuses on the notion that a mouse will spend more time researching and examining an object that it has never seen before (a novel object), as opposed to spending time researching and exploring an object that it has seen before (familiar object).

Apparatus

The apparatus is made up of a box that is open on all sides and measures 80 centimeters in width, 80 centimeters in length, and 50 centimeters in height. The items that needed to be distinguished were made of physiologically inert substances like glass, plastic, or metal. The objects under the study should be weight to prevent the animals from moving them.

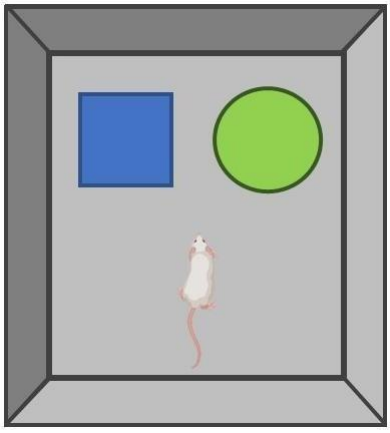


Figure 7: NOR test apparatus

Procedure

It comprises of two 10-minute test trials that are separated by 20-minute interludes. Five minutes were given for the participants to adjust to the square wooden box. Two comparable objects were put into the box being used to conduct the NOR test during the familiarization session, and the mouse was given free will to interact with the objects. One of the two familiar objects was taken

away after the familiarization session. A new object was put in the spot of the one that was removed. This new item was referred to as a novel object. The rat was free to investigate and engage with both objects after the inter-trial break. Rat physically touching or sniffing the object is regarded as exploration.

- Time spent with novel and familiar object.
- The discrimination index.

3.6.4 Morris water maze test (MWM)

Few years ago, Morris, 1981 introduced a device to examine the spatial learning and reference memory in rat. With repeated training exercises, animals discover the platform and discover how to get out of the pool. It has been used in some of the most complicated studies of the neurobiology and neuropharmacology of spatial learning and memory, despite the very simple core technique. (D'Hooge & De Deyn, 2001).

Apparatus

The tool was a circular steel pool with a height of 60 cm and a diameter of 120 cm. The pool was presumably divided into four quadrants and contained opaque water that was 34 cm deep. For the animals to exit the water, a submerged platform of 13 cm in diameter and 32 cm in height was positioned in the northwestern quadrant The animals might use spatial cues on the pool's walls to find a hidden platform by navigating from the release site around the pool's circumference.

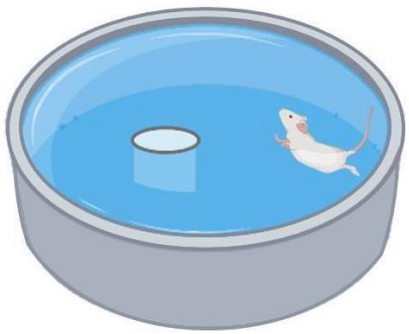


Figure 8: MWM test apparatus

Procedure

With a few minor alterations, the test protocol was the same as that previously described (Bromley-Brits et al., 2011). It features a five-day acquisition phase and a one-day probe trial after that. A total of five trials were conducted for the learning assessment each day. Throughout the whole test, the platform's location was unaltered. It was planned for the starting direction to change for every trial, every day (Table:2). The trial's cutoff time was 90 seconds, and there was a 10-minute break between trials. If the rat discovered the platform before the 90-second time limit, it was permitted to linger there for 5 seconds before being put back in its cage after drying. After 90 seconds, if the rat had not found the platform, it was placed there and given 20 seconds to remain there before being put back in its cage. The average of the five trials for each day was determined after the escape latency was recorded. On the sixth test day, one trial was conducted without a platform zone and with the release direction remaining unchanged. By monitoring the amount of time, the rat spent in the previously learned platform quadrant throughout the probing trial, reference memory was checked. Three parameters were calculated:

• Escape Latency over 5 days.

• Number of crossings over the platform on 6th day.

• Time spent in the target quadrant on 6th day.

Table 2 : Direction of release of rat, for Morris Water Maze Test

No. of Days	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
Day 1	West	South	North	East	South

No. of Days	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
Day 2	North	West	East	West	South
Day 3	North	East	West	South	North
Day 4	East	South	West	East	North
Day 5	West	South	North	East	South
Day 6	SINGLE TRIAL WITHOUT A PLATFORM (RELEASE DIRECTION WEST)				

3.7 Histopathological analysis of brain tissues

3.7.1 Perfusion fixation

As previously mentioned by (Gage et al., 2012), heart perfusion was carried out. Fixative solution was administered through the vascular system to fix tissue quickly and uniformly. The test mouse was put on the surgical table with its back facing down after being given anesthesia. Needles were driven through the appendages to hold them in place, securing the animal. By slicing through the connective tissues at the base of the diaphragm, the rib cage was made accessible. After that, the ribs were sliced just to the left of the midline of the rib cage. With the help of forceps, the heart was held steadily in a fixed position while a needle was introduced 5 mm deep into the left ventricular protrusion. The release of the perfusion drip valve allowed a slow, steady flow of 0.9% normal saline. To enable unrestricted solution flow through the vascular system, a cut was created in the right atrium with the use of sharp scissors. After passing saline, the body was free of blood. Instead of the saline, 4% paraformaldehyde (PFA)

solution was used then. The liver's coloration lightened after PFA injection, and the animal's tail stiffened. Perfusion was terminated at this moment, and the brain was carefully extracted. The isolated brain was treated for dehydration and embedding after being maintained in formaldehyde.

3.7.2 Paraffin embedding and block formation

The brain tissue was dehydrated over the course of 24 hours using a series of alcohols (isopropanol), 70% for 1 hour, 95% for 1 hour, and 100% for 1 hour, followed by paraffin infiltration. The brain tissues were next immersed in xylene for four hours. Paraffin embedding was then carried out by preserving the tissue in melted paraffin for 4 h at 60 °C, followed by four hours of cooling in a mould (block formation). After that, blocks were sectioned using a microtome at room temperature.

3.7.3 Hematoxylin and Eosin

On a 5µ tissue segment, conventional hematoxylin-eosin staining was carried out. The tissue was deparaffinized, incubated in Mayer's hematoxylin solution for 8 minutes, and then rinsed for 5 minutes in warm water. Eosin was used to counterstain the sections for a period of thirty seconds. Then soaking in 95% ethanol was done. Dry the slides and mount the cover slips. The images were seen under an inverted light microscope at magnifications of 4X, 10X, and 40X. OPTIKA Lite Software Version 2.11 was utilized for the acquisition and analysis of images.

3.8 Statistical analysis

Graphpad Prism version 8.0.1 was utilized in order to do the analysis on the results and to apply tests of One-way analysis of variance (ANOVA) or two-way ANOVA to dataset to determine whether statistical significance exists. Tukey's multiple comparisons test was also applied using

graphpad prism 8.0.1 to determine group to group differences. Mean ± SEM was used to present error bars.

CHAPTER 4

RESULTS

4.1 Phenotypic Identification of *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6

4.1.1 Colony Morphology

The purified bacterial isolates were cultured on MRS agar plates. These colonies were observed for their color and shape.

4.1.2 Gram staining

Gram staining was performed for the revived strain *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6

The strains were gram positive.

4.1.3 Catalase test:

No bubble formation was observed when *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6 were treated with 3% hydrogen per oxide. So, they were catalase negative.

4.2 Behavior Analysis

4.2.1 Effect of *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6 and Quercetin on anxiety in EPM

The Elevated Plus Maze (EPM) test was conducted to assess anxiety-like behavior in rats across different experimental groups, with a focus on their potential to prevent or delay Alzheimer's progression compared to an AlCl3-induced Alzheimer's disease model. The control group exhibited significantly lower anxiety-like behavior compared to the Diseased (D) group, with a p

value of <.0001. Both the Treated Probiotic and Quercetin group and the Treated Probiotic group alone displayed significantly reduced anxiety-like behavior compared to the DC group, with p values of <=.0001 and 0.0002, respectively. The Treated Quercetin group also demonstrated a significant decrease in anxiety-like behavior relative to the DC group, with a p value of 0.0180. These findings suggest that both probiotic and quercetin treatments effectively mitigate anxiety-like behavior in rats, highlighting their potential therapeutic relevance in the context of preventing or delaying Alzheimer's progression (Fig 9). Moreover, Statistical analysis revealed significant differences among the experimental groups. The control group exhibited a notably lower anxiety level compared to the diseased group ($p < .0001$), emphasizing the reliability of the EPM test in detecting anxiety-related behaviors. Treated groups with Probiotic and Quercetin jointly demonstrated a significant improvement compared to the diseased group($p = 0.0002$), showcasing the potential synergistic effect of the combined treatment. Additionally, individually, both Probiotic ($p = 0.0136$) and Quercetin ($p = 0.0429$) treatments exhibited statistically significant reductions in anxiety-like behavior compared to the diseased group. These findings underscore the potential therapeutic efficacy of Probiotic and Quercetin interventions in mitigating anxiety-related behaviors in rats and their potential for preventing or delaying Alzheimer's progression (Fig 10).

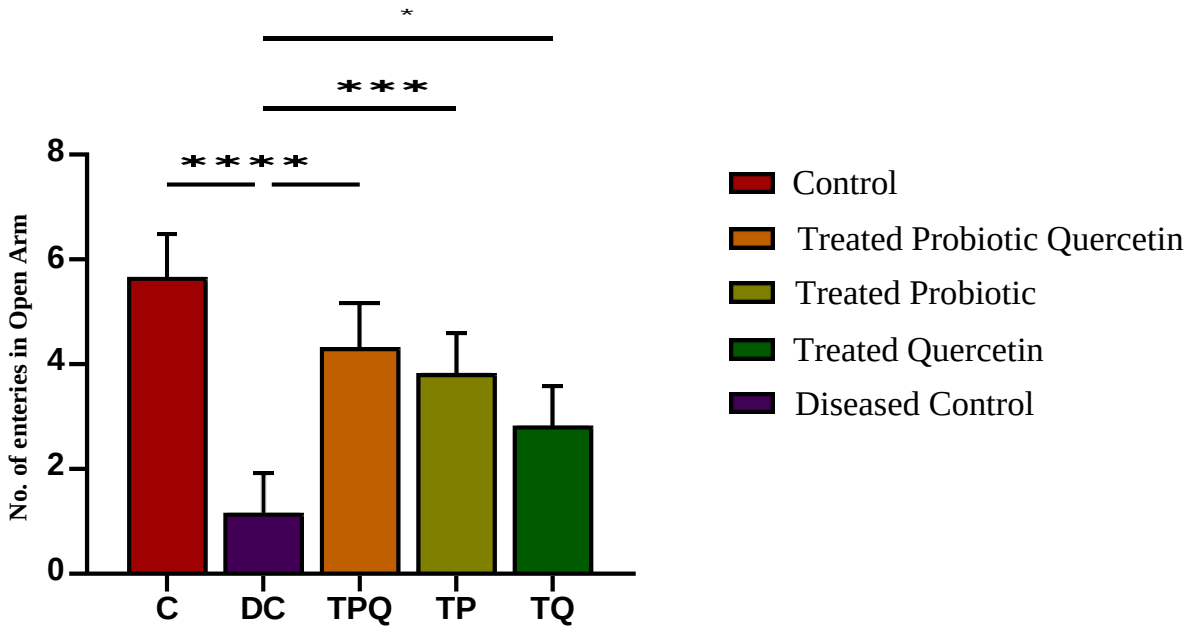


Fig 9: The effect of *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6 and Quercetin on Number of entries in Open arm.

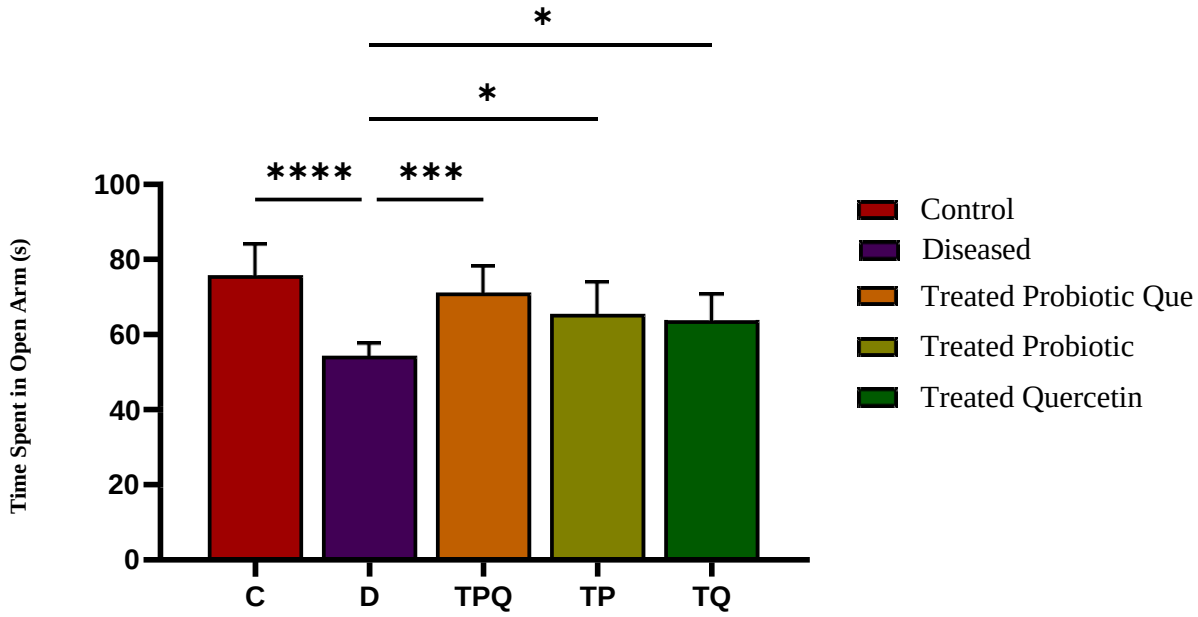


Fig. 10: The effect of *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6 & quercetin on Time spent in Open arm.

4.2.2 Effect of *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6 and Quercetin on spatial memory in Ymaze

In the Y-maze test evaluating rat behavior, exploration time in seconds provides significant insights. Both the Control Group and Diseased Group exhibit marked differences (p value < 0.0001) compared to the Treated Probiotic and Quercetin (TPQ) group in the "Other Arm," indicating notable alterations in behavior. Similarly, in the "Start Arm," distinct differences (p value < 0.0001) are observed between the Control Group and Diseased against TPQ. Notably, the crucial Novel Arm reveals compelling findings, with the Control Group significantly differing (p value < 0.0001) from the Diseased group. Furthermore, TPQ demonstrates remarkable superiority over the Diseased group (p value < 0.0001).

When examining the components of TPQ, Treated Probiotic significantly outperforms the Diseased group (p value < 0.0080), while Treated Quercetin also exhibits notable distinction (p

value < 0.0441). These results underscore the potential of TPQ in modifying rat behavior, suggesting promising therapeutic benefits in preventing or delaying the progression of Alzheimer's, particularly in the context of an AlCl3-induced Alzheimer's disease model (Fig. 11) Similarly, the evaluation of spontaneous alternation behavior in rats through the Y-maze test is critical for assessing cognitive function. The Control Group demonstrates significantly higher levels of spontaneous alternations compared to the Diseased group ($p < 0.0001$), indicating robust cognitive performance under normal conditions. Notably, the TPQ group shows a remarkable improvement in spontaneous alternations compared to the Diseased group ($p < 0.0003$), highlighting the potential cognitive benefits of combined treatment.

Moreover, both the Treated Probiotic and Treated Quercetin groups exhibit significant increases in spontaneous alternations compared to the Diseased group ($p < 0.0056$ and $p < 0.0066$, respectively), suggesting positive impacts on cognitive behavior. These findings collectively suggest the potential efficacy of probiotic and quercetin interventions in ameliorating cognitive deficits associated with Alzheimer's disease progression induced by AlCl3, as evidenced by the results of the Y-maze test (Fig. 12).

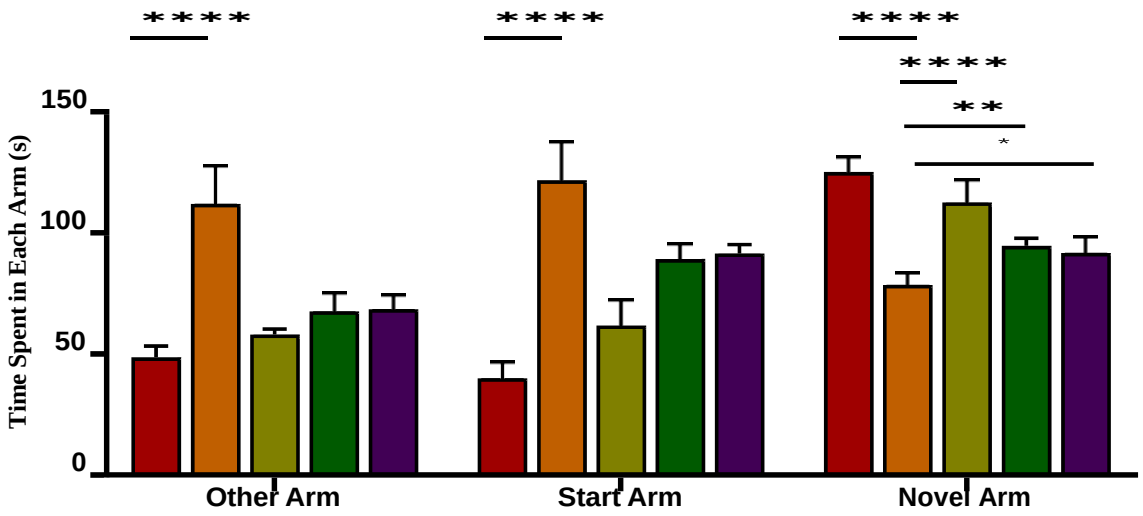


Fig 11: The effect of *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6 and Quercetin on Time spent in novel arm.

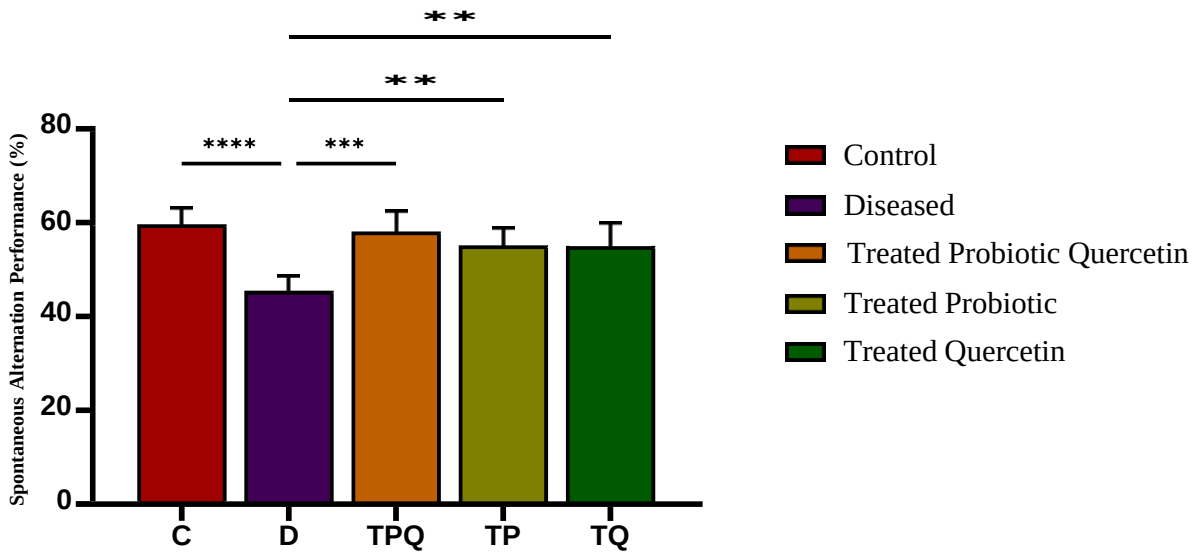


Figure 12: The effect of *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6 and Quercetin on (%) spontaneous alternation.

4.2.3 Effect of *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6 and Quercetin on recognition memory in NOR

In the novel object recognition test, exploration behavior and recognition memory were assessed by exposing test animals to various objects. Rodents typically exhibit a preference for exploring novel environments and objects over familiar ones, and the Discrimination Index (DI) was used to quantify recognition memory. Statistical analyses were performed to compare different groups, and the p-values were obtained. The results indicate a significant difference in recognition memory among the experimental groups.

The control group demonstrated a markedly lower DI compared to the Diseased group, with a p-value of < 0.0001, highlighting the impairment in recognition memory in the Alzheimer's disease condition induced by AlCl3. Treatment with both Probiotic and Quercetin yielded similarly impressive results, showing significantly higher DIs compared to the Diseased group, with p-values of < 0.0001. Additionally, when comparing individual treatments, both Treated Probiotic and Treated Quercetin groups exhibited substantially improved recognition memory compared to the Diseased group, with p-values of 0.0002 and 0.0123, respectively. These findings underscore the potential therapeutic impact of Probiotic and Quercetin interventions in preventing or delaying the progression of Alzheimer's disease in the AlCl3-induced Alzheimer's disease model (Fig. 13)

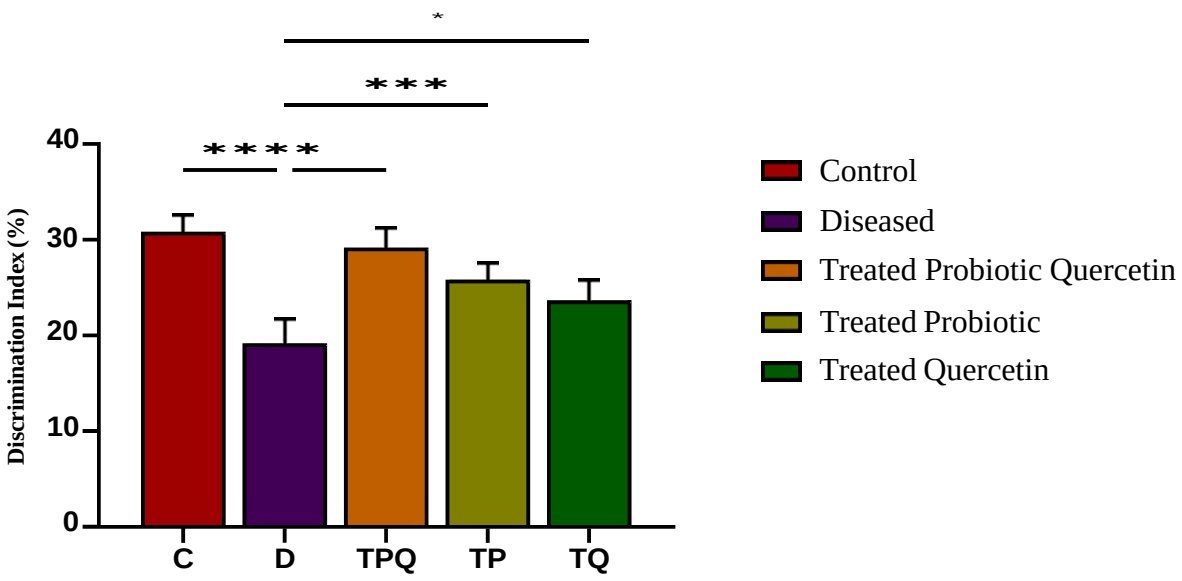


Figure 13: The effect of *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6 and Quercetin on recognition memory (% DI).

4.2.3 Effect of *L. fermentum* Y55 *and L. rhamnosus* Y59, *L. fermentum* FM6 and Quercetin on spatial learning & memory in MWM

The Morris Water Maze (MWM) serves as a robust tool for assessing spatial memory in rats through a two-phase protocol spanning six days. The examination involves a training phase and a subsequent probe trial. A pivotal metric in this evaluation is the time spent by rats in the target quadrant. In the presented study, distinct groups were analyzed: a Control group, a Treated Probiotic and Quercetin group, and a Treated Probiotic group. The results, as evidenced by the time spent in the target quadrant, showcased significant variations.

The Control group exhibited a noteworthy disparity compared to the AlCl3-induced Alzheimer's disease model (Diseased group) ($p < 0.0001$). Meanwhile, the Treated Probiotic and Quercetin group demonstrated a remarkable difference from the Diseased group ($p = 0.0002$). Additionally, both the Treated Probiotic and Treated Quercetin groups displayed significant distinctions in

comparison to the Diseased group (p = 0.0012 and p = 0.0126, respectively). These findings underscore the potential cognitive benefits of probiotic and quercetin treatments in mitigating spatial memory deficits associated with Alzheimer's disease progression induced by AlCl3, as evidenced by the MWM results (Fig. 14).

Similarly, Assessment of platform crossings, a key metric, revealed significant findings. The Control group demonstrated remarkable efficacy, as indicated by a p value of <.0001 compar ed to the Diseased D group. Treated groups, involving Probiotic and Quercetin, exhibited equally promising outcomes with p values of ≤0.0001 against the Diseased D Group. Notably, Treated Probiotic alone displayed significance (p value = 0.0331), while Treated Quercetin showed efficacy with a p value of 0.0049 against the Diseased group. These findings underscore the potential therapeutic impact of the interventions on spatial memory in rats and suggest their potential in preventing or delaying Alzheimer's progression induced by AlCl3 (Fig. 16)

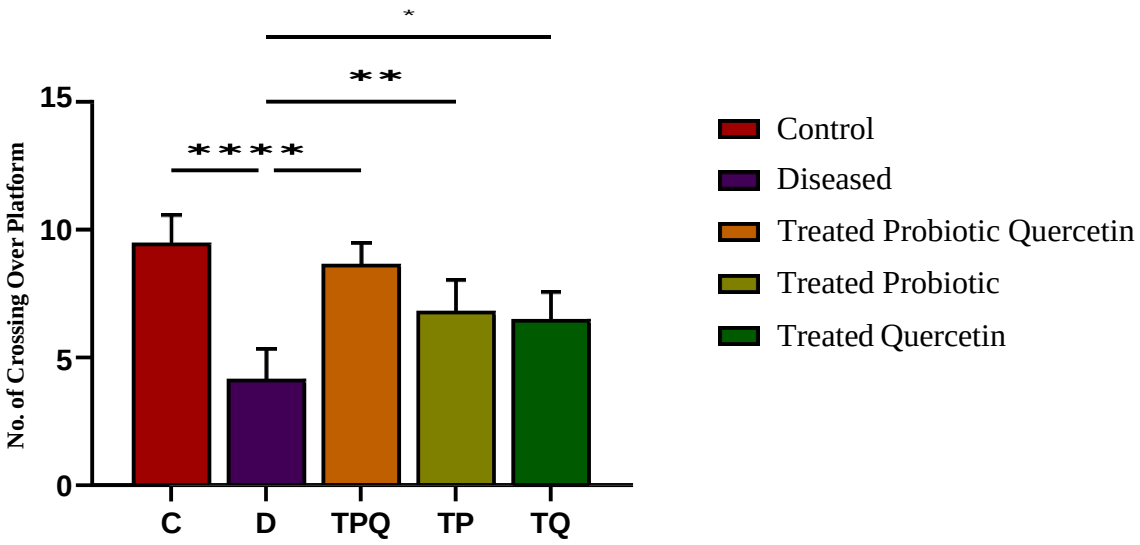


Figure 14: The effect of *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6 and Quercetin on spatial memory in MWM.

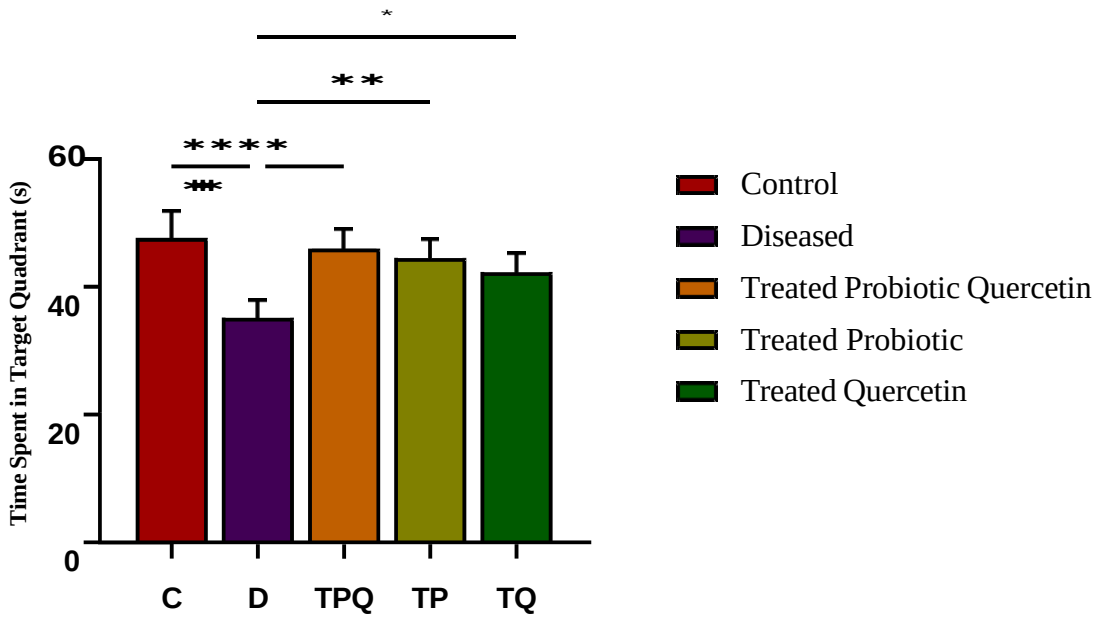
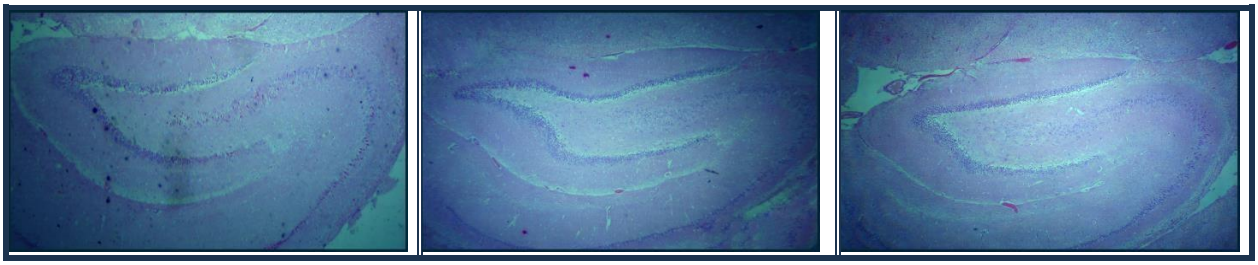


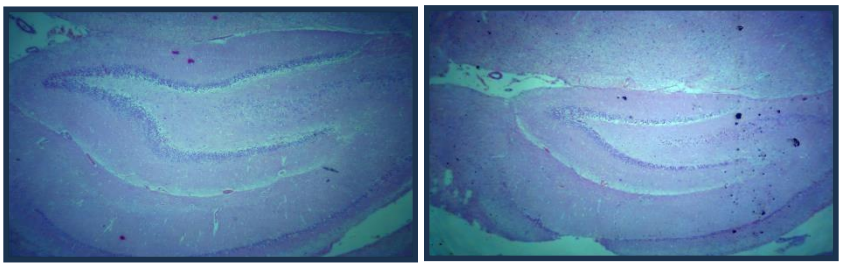
Figure 15 : The effect of *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6 and Quercetin on time spent in target quadrant in MWM .

4.3 Histopathological assessment

The hippocampus of rat brain was stained with hematoxylin and eosin stain. Results of staining showed that neurodegeneration was observed in diseased D group in hippocampus as compared to the negative control. Combination treatment of *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6 and Quercetin have significantly restored neurodegeneration in AlCL3-induced model showing neuroprotective effect of both. On the other hand, probiotic treatment TP group and quercetin treatment TQ group also showed comparable better results than diseased group.

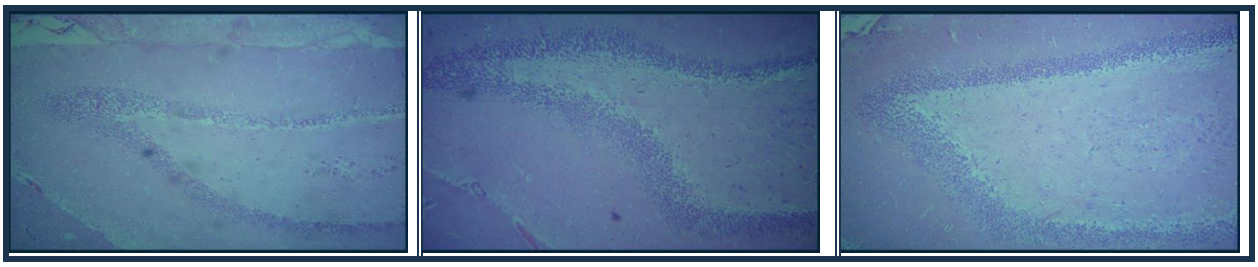


(A) **(B)** **(C)**

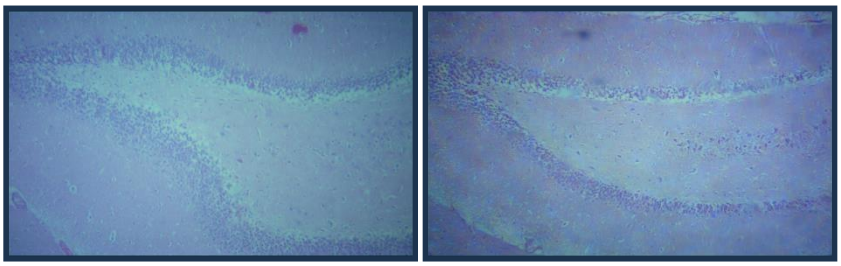


(D) **(E)**

Figure 16: H&E stained coronal sections of hippocampus 4X magnification. (A) Control (B) *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6 and Quercetin treatment TPQ Group , (C) Probiotics Treated Group TP (D) Quercetin Treated Group TQ , (E) Alcl3 induced diseased D group



(A) **(B)** **(C)**



(D) **(E)**

Figure 17: H&E stained coronal sections of hippocampus 10 X magnification. (A) Control (B) *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6 and Quercetin treatment TPQ Group , (C) Probiotics Treated Group TP (D) Quercetin Treated Group TQ , (E) Alcl3 induced diseased D group

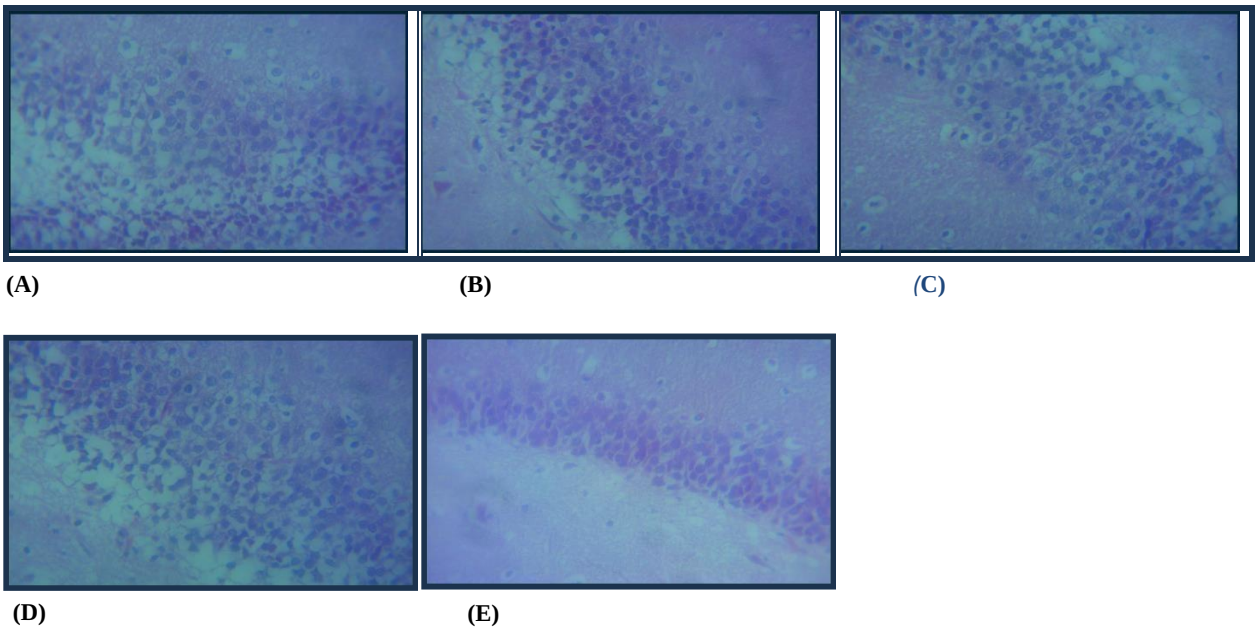


Figure 18: H&E stained coronal sections of hippocampus 40 X magnification. (A) Control (B) *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6 and Quercetin treatment TPQ Group , (C) Probiotics Treated Group TP (D) Quercetin Treated Group TQ , (E) Alcl3 induced diseased D group.

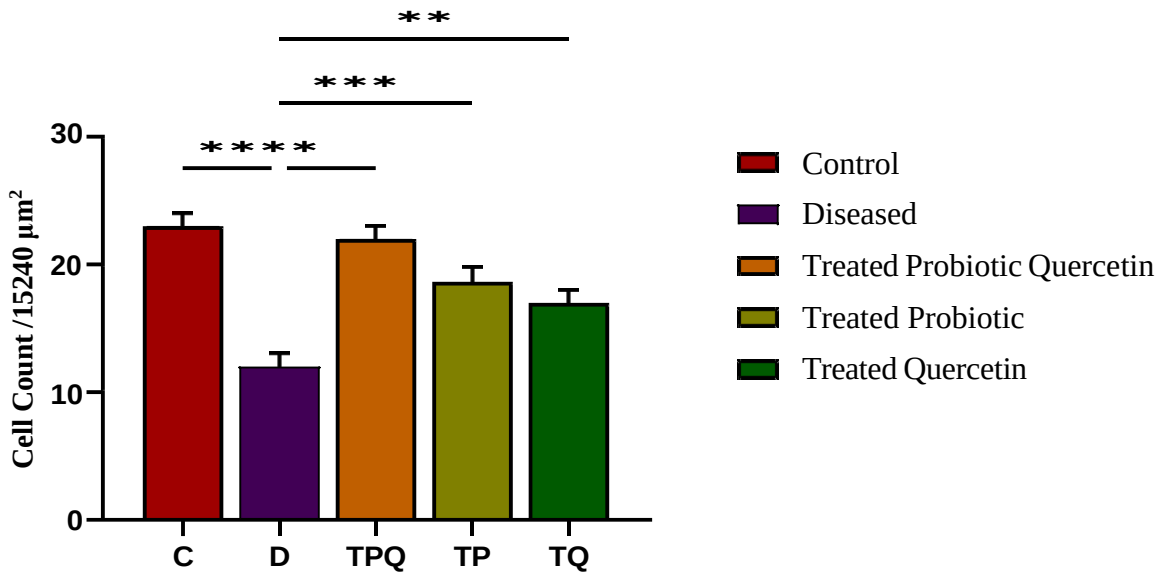


Figure 16: The effect of *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6 & Quercetin on cell count in dentate gyrus, hippocampus

CHAPTER 5

DISCUSSION

Alzheimer's disease is a chronic, multifactorial, age-related illness for which there is no one cause that can be identified. Memory storage and retrieval are impacted by Alzheimer's disease. No single pharmaceutical product is able to simultaneously target all of While these risk factors are diverse, it will be difficult to successfully stop the disease from progressing unless these risk factors are addressed. This is due to the fact that no single risk factor has this ability. The development of Alzheimer's disease can be influenced by several variables. Neuroinflammation, metabolic instability, increased oxidative stress, and an imbalance in neurochemical signalling are a few of these variables. Similarly, the treatments currently available for Alzheimer's disease only target specific symptoms like amyloid beta and neurofibrillary tangle accumulation, memory loss, behavioural issues, abnormalities in acetylcholine levels, or changes in sleep patterns (Broadstock et al., 2014).

In this study, Probiotic Isolate *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6, were selected that were previously isolated by lab fellow Noor, (2019) and Hafsa Raja, (2022). These strains were further characterized exhibiting antioxidant potential by in-silico analysis done by another lab fellow Ayesha, (2022). Because of their potential as an antioxidant, these probiotic strain acts as a buffer against oxidative stress, which can cause AD. Since Alzheimer's disease is associated with increased oxidative stress, amyloid beta plaques build up. Numerous possible advantages of *Limosilactobacillus fermentum* Y55, *Lacticaseibacillus rhamnosus* Y59, *Limosilactobacillus fermentum* FM6, include the maintenance of gut microbiota, the lowering of cholesterol, and the treatment of liver illnesses. Probiotics such as Limosilactobacillus fermentum and *Lacticaseibacillus rhamnosus*, have been

used as an AD treatment in this particular study. Additionally, a polyphenol known as quercetin is included to boost the antioxidant action; these two components combined provide a symbiotic composition. Moreover, possessing antioxidant and anti-Alzheimer's properties, quercetin is a useful strategy for combating prevalent cognitive impairments such as Alzheimer's.

The results of the present study provide valuable insights into the potential therapeutic effects of Limosilactobacillus fermentum Y55, Lacticaseibacillus rhamnosus Y59, Limosilactobacillus fermentum FM6, and Quercetin in an aluminum chloride (AlCl3)-induced Alzheimer's rat model. The study employed a multidimensional approach, combining microbiological, behavioral, and histopathological assessments to comprehensively evaluate the impact of the interventions.

The initial sections of the study focused on the characterization of the selected probiotic strains, emphasizing the importance of a rigorous selection process based on in-silico analysis and previous isolation and characterization work. The use of high-quality chemicals and standards in the study, such as aluminum chloride hexahydrate, MRS Broth, and MRS Agar, ensures the reliability and reproducibility of the experimental procedures.

The behavior studies conducted on Wistar rats encompassed a range of tests to assess anxiety, spatial memory, recognition memory, and overall cognitive function. The Elevated Plus Maze (EPM) test revealed a significant reduction in anxiety-like behavior in both the Probiotic Treated group and the Quercetin Treated group compared to the Diseased group. Moreover, the combined treatment group (Probiotic & Quercetin) demonstrated a synergistic effect, further reducing anxiety levels. These findings align with previous research indicating the anxiolytic potential of probiotics and natural compounds like quercetin.

The Y-maze test provided insights into spatial memory and spontaneous alternation behavior. The results indicated a notable improvement in spatial memory in both the Probiotic Treated group and

the Quercetin Treated group compared to the Diseased group. The combined treatment group exhibited enhanced performance, underscoring the potential synergistic cognitive benefits of probiotics and quercetin. Spontaneous alternation, a marker of cognitive flexibility, also improved significantly in treated groups compared to the Diseased group.

The Novel Object Recognition (NOR) test further supported the cognitive benefits of the interventions, with both Probiotic Treated and Quercetin Treated groups displaying higher Discrimination Index (DI) values compared to the Diseased group. These findings suggest an improvement in recognition memory, a crucial aspect affected in Alzheimer's disease.

The Morris Water Maze (MWM) test, a gold standard for assessing spatial learning and memory, demonstrated significant improvements in the Probiotic Treated group and Quercetin Treated group compared to the Diseased group. The time spent in the target quadrant, the number of crossings over the platform, and other parameters collectively highlighted the cognitive-enhancing effects of the interventions.

Histopathological assessment of the rat hippocampus provided crucial insights into the neuroprotective effects of the treatments. The AlCl3-induced diseased group exhibited neurodegeneration, while the Probiotic & Quercetin Treated group showed significant restoration of neurodegeneration. Both individual treatments (Probiotic Treated and Quercetin Treated) also demonstrated neuroprotective effects, further supporting the cognitive benefits observed in the behavioral tests.

The study's strength lies in its holistic approach, integrating microbiological, behavioral, and histopathological evaluations. The results collectively suggest that the selected probiotic strains and quercetin have the potential to mitigate the cognitive deficits associated with Alzheimer's

disease. The synergistic effects observed in the combined treatment group indicate a promising avenue for future research and therapeutic interventions.

However, it is essential to acknowledge certain limitations of the study. The animal model used, while providing valuable insights, may not fully replicate the complexity of Alzheimer's disease in humans. Additionally, the specific mechanisms underlying the observed effects require further investigation. Future studies could delve into the molecular and biochemical pathways involved in the neuroprotective and cognitive-enhancing effects of the probiotic strains and quercetin.

Thus, this study contributes to the growing body of research exploring alternative therapeutic approaches for Alzheimer's disease. The promising outcomes observed in the behavioral tests and histopathological assessments suggest that Limosilactobacillus fermentum Y55, Lacticaseibacillus rhamnosus Y59, Limosilactobacillus fermentum FM6, and Quercetin may hold potential as complementary interventions in the management of Alzheimer's disease. Further research, including clinical trials, is warranted to validate these findings and translate them into potential therapeutic strategies for human patients.

Chapter 7

Conclusion And Future Prospects

The current study showed that daily consumption of a combination of probiotics and quercetin has been observed to slow down the progression of Alzheimer's disease. Moreover, the combined administration of probiotics and polyphenols has shown promise in enhancing cognitive function related to Alzheimer's and reducing anxiety-like behavior, surpassing the effects observed with the separate use of probiotics or polyphenols alone.

In Future, Analyzing fecal microbial diversity through 16S RNA profiling offers valuable insights into how oral consumption of probiotics and polyphenols influences gut microbe variety. Real-time RT-PCR enables assessment of antioxidant enzyme activity, providing further understanding of potential health benefits. However, additional clinical studies are necessary to validate these advantages. Furthermore, the formulation of probiotics and polyphenols into food products holds promise for commercialization, potentially offering convenient and effective ways to improve gut health and overall well-being.

CHAPTER 6

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NUST-IRB Certificate

1. Research Project Title: Investigating the effect of indigenous probiotic Lactobacillus spp. Strains and Quercetin on AlCl3 induced Alzheimer's disease rat model.

2	Name of PI:	Dr Abdul Rahman
3	Duration:	03-04 months
4	Name of Institution / Department	ASAB, NUST
5	IRB No.	07-2022-ASAB-01/02

6. The project proposal entitled above has been reviewed by the NUST Institutional Review Board Meeting held on July 01, 2022.

7. The Board approves project proposal on scale and criteria given below to be implemented before/during project execution.

- Safety Measures
- Workspace Requirements
- Protection from potential hazards & Risks
- Confidentiality Requirements (If Any)

Note: The Ethical Review Committee reserves the rights to re-review the project during the project execution to address the suggested guidelines.

Prof. Dr. Muhammad Tahir
Head, Thematic Research

Signature of the Head

Pro-Rector (RIC)
National University of Sciences and Technology
(Dr. Rizwan Riaz)

Signature & Seal of The Chairman NUST-IRB

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Chapter 4 Results

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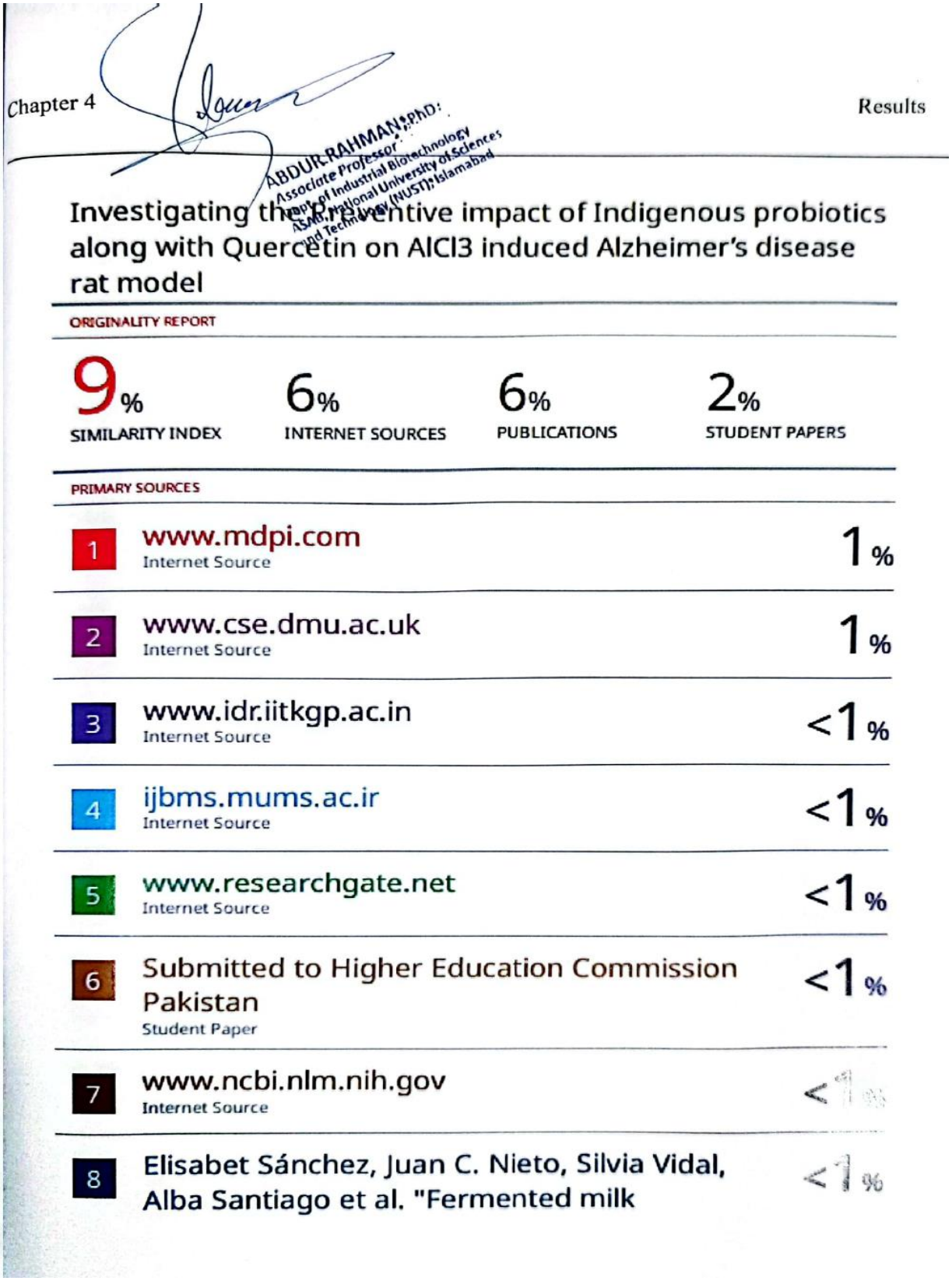

Chapter 4 Results

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Investigating the Preventive impact of Indigenous probiotics along with Quercetin on AlCl3 induced Alzheimer's disease rat model

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