Elucidation of Therapeutic Effects of Probiotics in Mouse Model of Alzheimer's Disease

Master of Science

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2022

Elucidation of Therapeutic Effects of Probiotics in Mouse Model of Alzheimer's Disease

A thesis submitted in partial fulfillment of the requirement for the degree of Masters of Science

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This thesis is dedicated to my parents for their endless love and support

ACKNOWLEDGEMENTS

In the name of Almighty Allah, the Most kind, the Most merciful.

I would like to extend my highest gratitude to my supervisor Dr. Saadia Zahid for the opportunity to work in her lab and under her guidance, for her advocacy and for inspiring me to be the best researcher through her proficiency and knowledge. This project could not have been completed without her guidance.

I am thankful to Dr. Hussnain A. Janjua, Principal ASAB and Dr. Sobia Manzoor, HoD Healthcare Biotechnology for their academic support. I am also grateful to my GEC members Dr. Abdur Rehman (ASAB, NUST), Dr. Saira Justin (ASAB, NUST) and Dr. Saima Zafar (SMME, NUST) for their guidance throughout my research journey.

A special thank you to my lab assistant Ms. Shumaila, my lab mates particularly Sumaiya Mansoor, and my seniors Aymen Tahir, Nishat Malik, Humna Asghar, Sara Ishaq and Dr. Sanila Amber. Thank you to my wonderful friends Amna Hafeez, Khired Sajid, Murtaz Ahmad and Tauheed Ahmad for their endless moral support and encouragement.

I am extremely grateful to my parents without whom this achievement would not have been possible. Their endless love, support and prayers have always pushed me forward. I would not be the person I am today, without their strength and guidance. I am also grateful to my siblings, Muzammil and Maleeha for always cheering me on. I would like to express my heartfelt gratitude to my husband Jiyad-ur-Rehman for having faith in me and my abilities.

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LIST OF ABBREVIATIONS

µ	Micro
Aβ	Amyloid beta
ACE	Acetylcholinesterase
AD	Alzheimer's disease
$AlCl_3$	Aluminium Chloride
AP	Anteroposterior
APOE	Apolipoprotein E
APP	Amyloid Precursor Protein
BBB	Blood Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
cDNA	Complementary DNA
CSF	Cerebrospinal Fluid
DSM- -IV	The Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition
DV	Dorsoventral
EPM	Elevated Plus Maze
GABA	Gamma amino-butyric acid
GBA	Gut Brain Axis
GF	Germ Free
GIT	Gastrointestinal Tract
GSK-3	Glycogen synthase kinase 3
HPA	Hypothalamic-Pituitary-Adrenal

IL-1β	Interleukin-1β
IL-8	Interleukin-8
IMH	Italian Ministry of Health
IRB	Internal Review Board
LPS	Lipopolysaccharide
MCI	Mild Cognitive Impairment
ML	Mediolateral
MRI	Magnetic Resonance Imaging
MWM	Morris Water Maze
NFT	Neurofibrillary Tangle
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association
NOR	Novel Object Recognition
PBS	Phosphate Buffer Saline
PET	Positron Emission Tomography
PFA	Paraformaldehyde
PP-2A	Protein Phosphatase-2A
PS-1	Presenilin-1
PS-2	Presenilin-2
RT-PCR	Real Time-Polymerase Chain Reaction
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species

SCFA	Short Chain Fatty Acid
SPF	Specific Pathogen-Free
TNF-α	Tumor Necrosis Factor-α

ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder associated with age-related cognitive decline. It characterized by a host of neurological and psychiatric symptoms particularly learning and memory deficits. Currently, AD has no treatment that halts the progression of the disease. The gut microbiota modulates gut brain axis by facilitating development of hypothalamic-pituitary-adrenal axis and synthesis of neuromodulators such as GABA, SCFAs, serotonin and BDNF. This study investigated the effect of oral consumption of probiotics; *Lactobacillus rhamnosus* GG (LGG®) and *Bifidobacterium* BB 12 (BB-12®) ($1x10^9$ CFU) on $AlCl_3$ -induced AD mouse models in comparison with Donepezil. Mice were randomly allocated to six different study groups (n=8). Behavioral tests were conducted to assess effect of $AlCl_3$ (300mg/kg) and probiotics treatment on anxiety and memory through Elevated Plus Maze (EPM), Y-maze, Morris Water Maze (MWM) and Novel Object Recognition (NOR) test. The results indicated that probiotic treatment significantly ($p<0.0001$) reduced anxiety post $AlCl_3$ exposure. The $AlCl_3$ + LGG®& BB-12® treated group showed significantly ($p<0.0001$) improved spatial memory in comparison to $AlCl_3$-treated group. Also, significant improvement ($p<0.0001$) was observed in recognition memory in $AlCl_3$ + LGG®& BB-12®-treated group in comparison to $AlCl_3$-treatedgroup, only. The $AlCl_3$ + LGG®& BB-12®-treated group also exhibited significant improvement ($p<0.0001$) in spatial memory as compared to $AlCl_3$-treatedgroup, only. Histopathological assessment performed through Congo red staining showed remarkable decrease in amyloid plaque burden within the mice hippocampus observed in the group treated with LGG®& BB-12® post $AlCl_3$ exposure. Effects of selected probiotics on the expression of inflammatory cytokines i.e., TNF-α and IL-1β

was also evaluated through real time PCR. Differential expression of TNF-α and IL-1β was observed in controls and $AlCl_3$-treated group. Whereas the LGG®& BB-12® treatment post $AlCl_3$ exposuresignificantly decreased ($p<0.0001$) the expression of the inflammatory cytokines as compared to $AlCl_3$-treatedgroup, only. The present findings indicate that probiotics like LGG®& BB-12® have strong potential to be used as combination therapy for AD by modulating the gut microbiota-brain axis.

INTRODUCTION

Introduction

Alzheimer's disease (AD) is a senescence-related neurodegenerative disorder that is irreversible (Marino et al.,2019). The major hallmark of this disease is memory loss along with a plethora of different cognitive challenges such as impairment in decision making and judgment, language difficulties and inability to recognize common objects (Breijyeh&Karaman, 2020). In conjunction with these symptoms, AD patients also exhibit neuropsychiatric symptoms such as psychosis, anxiety, aggression and apathy (Li et al., 2014).

AD is a daunting public health concern contributing heavily to disease burden. An estimated 50 million people suffer from AD globally (Zhang et al., 2021). A major chunk of diagnosed dementia cases, roughly 60-80% are due to AD (Alzheimer's disease facts and figures, 2020). It is a cause of financial distress costing up to $1 trillion dollars annually for patient care (World Alzheimer's Report, 2016).

The disease is complex in nature with a complicated multifactorial pathophysiology. The four major hypotheses of AD pathology are amyloid β (Aβ) deposition cascade, tauopathy, oxidative stress and inflammation. β- secretases and γ-secretases cleave the amyloid precursor protein leading to formation of oligomers that form deposits within the brain (Stanciu et al., 2020). Hyperphosphorylation of tau protein by impaired activity of protein kinases and phosphatases leads to tau protein with lower binding affinity for microtubules. This hyperphosphorylated tau leads to formation of neurofibrillary tangles (Liu et al., 2020). An increase in oxidative stress by unquenched reactive oxygen species leads to cell damage. These include superoxide, hydrogen peroxide, hydroxyl ion and

peroxynitriteanions. ROS are produced under normal physiological conditions and are involved in metabolism and cell signaling. However, excessive production of ROS leads to cell damage and neuronal death (Stefanatos&Stanz, 2018). The progression of AD is also mediated by inflammation. Activated glial cells such as microglia and astrocytes within the brain express chemokine and cytokines that lead to inflammation (Wand &Xie, 2022).

The enteric nervous system and CNS maintain a communication pathway between them called gut brain axis (GBA). The GBA links the brains functions of cognition and emotion to the peripheral functions of intestine. The gut microbiota is an important variable of GBA (Dinan&Cryan, 2017). The gut microbiota is a complex microbial community with bacteria contributing majorly to the population with other minor contributors such as fungi, viruses, protozoa etc. Bacteria of phyla Bacteroidetes, Firmicutes and Proteobacteria mainly contribute to the microbial composition of gut and are present in the highest relative abundance (Grenham et al., 2012).

Host and Gut microbiota are symbiotic in nature. They promote regeneration of intestinal epithelial cells, promote intestinal mucosal integrity, stimulation and maturation of immune system, metabolism of nutrients and synthesis of hormones and vitamins (Nell &Josenhans, 2010; Jandhyala et al., 2015). The hypothalamic-pituitary-adrenal axis, vagus nerve and neurotransmitters or neuromodulators like short chain fatty acids, hormones such as ghrelin, leptin and gases such as CO_2, CO, H_2S, and NH_3act as pathways of communication between gut microbiome and brain (Hill et al., 2014).

Gut microbiota of AD patients exhibits dysbiosis with a decrease in Firmicutes and increase in Bacteroidetes and Proteobacteria (Liu et al., 2019). This evidence along with

evidence of microbiota in gut health and modulation of brain function, indicates that modulating diversity of gut microbiota may serve as a potential route for therapy.

Current treatments available for AD are acetylcholinesterases such as donepezil, rivastigmine etc. They provide symptomatic treatment and do not halt the disease progression. Furthermore, they are associated with side effects ranging from moderate severity such as vomiting and weight loss to high severity such as bradycardia and insomnia (Joe &Ringman, 2019).

In lieu of this evidence, a novel approach is required for the treatment of AD. Living microorganisms that colonize the gut of host and confer health benefits are called probiotics. Benefits conferred are dependent on specific probiotic species. They are well-tolerated, highly accessible and require low production costs, making them good potential candidates for treatment of AD.

1.1 Aim and objectives

The present study was conducted to determine the therapeutic effects of oral administration of lyophilized probiotic formulation of *Lactobacillus rhamnosus* LGG and *Bifidobacterium lactis* BB-12®on the cognitive deficits and inflammatory markers observed in AD mouse model as compared to standard drug donepezil.

Objectives include:

- To analyze the effects of probiotic consumption on cognitive deficits of learning and memory displayed by AD mouse model via behavioral tests.
- To explicate the effect of probiotics on proinflammatory cytokines by quantifying the mRNA expression of TNF-α and IL-1β.
- To compare the effect of donepezil and probiotic treatment on neurodegeneration in AD mouse model.

LITERATUREREVIEW

2.1 Alzheimer's Disease

AD is an age-related, irreversible neurodegenerative disease, marked by wide spread neuronal loss and atrophy of gray and white matter resulting in brain volume loss (Bartzokis, 2011; Marino et al., 2019). Brain regions most affected by cerebral atrophy include the hippocampus, cingulate gyrus, prefrontal cortex, parietal and temporal lobes (Traini et al., 2020).

AD is a considerably significant public health concern and affected over 50 million people worldwide in 2020 (Zhang et al., 2021). The number is anticipated to exceed 131 million individuals by 2050. AD accounts for roughly 60-80% of diagnosed dementia cases (Alzheimer's disease facts and figures, 2020). Not only is the disease debilitating for patients but it is also financially crippling. Roughly $1trillioncost was incurred for healthcare and long-term care for AD patients in 2018 (World Alzheimer's Report, 2016). It is also detrimental to the emotional and physical health of primary caregivers of AD patients. A study reported that 37% of nursing staff attending to patients suffering from AD self-reported as experiencing high emotional burden and 32% reported high physical burden (Albers et al., 2014).

Although AD is primarily associated with age-related cognitive decline but several risk factors exist that influence incidence of disease. Risk factors can be split into three categories; genetic risks, co-morbidities and lifestyle risks (Edwards III et al., 2019). Genetic risk factors for familial AD include amyloid precursor protein (APP), presenilin 1 (PS-1), or PS-2 (Ryman et al., 2014). These genes lead to increase in expression of Aβ

in neuronal cells. Apolipoprotein E (APOE) gene particularly the epsilon4 allele also serves as major risk factor (Lane et al., 2018).

Co-morbidities that serve as risk factors include cerebrovascular diseases such as hypertension or cardiac arrest, type 2 diabetes mellitus, depression, obesity, epilepsy or traumatic brain injury (Li et al., 2015; Cretin et al., 2016). Modifiable lifestyle risk factors are frequency and intensity of exercise, sleep disturbances, alcohol consumption, smoking and low education (Winter et al., 2007; Bonanni et al., 2005; Heymann et al., 2016). AD also exhibits gender and sex differences with females experiencing two-fold higher risk of incidence than males (Podcasy & Epperson., 2016).

Diagnosis of AD is based on Diagnostic and Statistical Manual of Mental Diseases (DSM- IV) criteria for Alzheimer specific dementia andNational Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria for probable AD (Bature et al., 2017). The diagnosis is further confirmed by biomarkers such as increased phosphorylated tau proteins in cerebrospinal fluid (CSF), loss of volume in hippocampus and shrinkage of brain size on structural magnetic resonance imaging (MRI) or presence of amyloid fibroids in positron emission tomography (PET)(Neugroschl & Wang., 2011; Morbelli & Bauchkneht, 2018).

As AD is a multifactorial disease with no definitive pathway for disease onset and progression, the treatment aims to curtail symptoms severity. Decrease in density of cholinergic neurons is a preliminary hallmark of AD, therefore inhibition of acetylcholinesterase(ACE) which recycles synaptic acetylcholine in gray matter, is the

most favored strategy. Inhibition of acetylcholinesterase results in prolonged acetylcholine activity (Weller &Budson, 2018).

Commonly used ACE inhibitors are donepezil, galantamine and rivastigmine. These drugs are administered orally and have a wide range of adverse effects including nausea, vomiting, loss of appetite followed by weight loss, heart block, bradycardia, insomnia, dizziness, allergic dermatitis and muscle cramps etc (Joe & Ringman, 2019). This list of adverse effects is by no means exhaustive. Thus, new interventions must be explored that mitigate disease progression and have fewer adverse effects.

2.2 Pathophysiology of AD

Several different biological processes contribute to the pathophysiology of AD, which makes it as complex and multi-factorial disorder. The different pathways include Aβ-cascade, tauopathy, inflammation and reactive oxygen species (ROS). These pathways are involved in disease onset and progression. These pathways are not isolated instead they interact and exacerbate one another. An overview of AD pathophysiology is provided in the section below (Jagust, 2020).

Aβ-cascade is the most widely accepted hypothesis for AD pathology. The APP undergoes proteolytic cleavage to give rise to Aβ that constitutes amyloid deposits in brain (Puig & Combs, 2013; Macleod et al., 2015). APP undergoes cleavage via three enzymes; β- secretase, γ-secretase and α-secretase at the N-terminal, C-terminal and within Aβ domain respectively. Oligomers produced by β- secretase and γ-secretase may aggregate and form senile plaques (Stanciuet al., 2020). The onset of the disease is thought to be triggered by Aβ accumulation (Dunys et al., 2020).

Tau protein is a microtubule-associated phosphoprotein that constitutes neurofibrillary tangles (NFTs) found in hippocampus of AD brains. Itis involved in maintaining cell stability and supporting axonal transport (Alonso et al., 2018). The expression of tau protein is reportedly 7x higher in neocortex of AD patients. The hyperphosphorylation of tau protein leads to its reduced binding ability to microtubules. This results in a disturbance in axonal transport and cell stability. These abnormally hyperphosphorylated tau proteins form inert polymers called neurofibrillary tangles (Liu et al., 2020).

Glycogen synthase kinase-3 (GSK-3) which is a protein kinase and phosphatases like phosphatase 2A (PP-2A) are involved in abnormal phosphorylation of tau. GSK-3 induces a highly immunoreactive in neurons of AD patients. It modulates synaptic plasticity and cytoskeletal processes (Kirouac et al., 2017). PP-2A is reported to have lower activity in brains of AD patients (Wei etal., 2020).

Mitochondria generates a high amount of energy in the brain for functions such as neuronal membrane potential, neurotransmitter synthesis and release and axonal transport which contributes to production of reactive oxygen species (ROS) (Hyder et al., 2013). ROS contributes to maintenance of normal physiology by activating pro-inflammatory mechanisms, cell survival and response to stress (Sies & Jones, 2020). However, unquenched ROS leads to cell damage (Rangaraju et al., 2014). Aβ and tau protein accumulation further enhances oxidative stress. Increased accumulation of ROS causes damage to neuronal membranes, nucleus and mitochondrial DNA and loss of synaptic plasticity (Stefanatos& Sanz, 2018)). Perrotte et al., 2019 reported a marked decline in total antioxidant capacity in plasma of AD patients which positively correlated with scores of MoCA and MMSE (Perrotte et al., 2019) Aβ deposition and cell damage caused

by ROS and NFTs lead to activation of inflammatory response in brain. Inflammatory response involves astrocytes, microglia and lymphocytes that release cytokines, proinflammatory cytokines and ROS (Wang &Xie, 2022). Microglia are primary form of immune defense within the CNS. They have neuroprotective functions but may also be neurotoxic. Microglia secretes IL-1β, TNF-α, IL-6, and IL-8. They increase the permeability of BBB to peripheral monocytes (Wang et al., 2015). Chronic microglia activation leads to neuroinflammation and neurotoxicity. Astrocytes are glial cells that provide nutrients and heal injuries to brain and spine. They release chemokines that attract microglia leading to a proinflammatory response (Gonzalez-Reyes et al., 2017).

2.3 Gut Brain Axis

A pathway of communication exists between enteric nervous system and CNS called the gut brain axis. This pathway is bi-directional in nature. It links the peripheral intestinal functions such as secretion of digestive enzymes and mucous, absorption of water, electrolytes and nutrients etc. to the brain's emotional and cognitive centers. The microbiome of gut is an important player of the GBA and therefore has also been termed as gut microbiota-brain axis. Since the microbiota interacts with the brain through various pathways, the microbiota is considered an independent variable in GBA (Rhee et al., 2009; Dinan & Cryan, 2017).

Gut microbiota is a complex functional microbial community that inhabits the gastrointestinal tract (GIT) of humans and has a symbiotic relationship with its hosts. There are an estimated of 1×10^{13} to 1×10^{14}microbes in the GIT. The abundance of microbes is 10× that of average number of cells in host body (Sender et al., 2016). The

microbial community in GIT predominantly consists of anaerobic bacteria; other microbes such as fungi, viruses, archaea and protozoa contribute a smaller percentage to the overall diversity (Wampach et al., 2017).

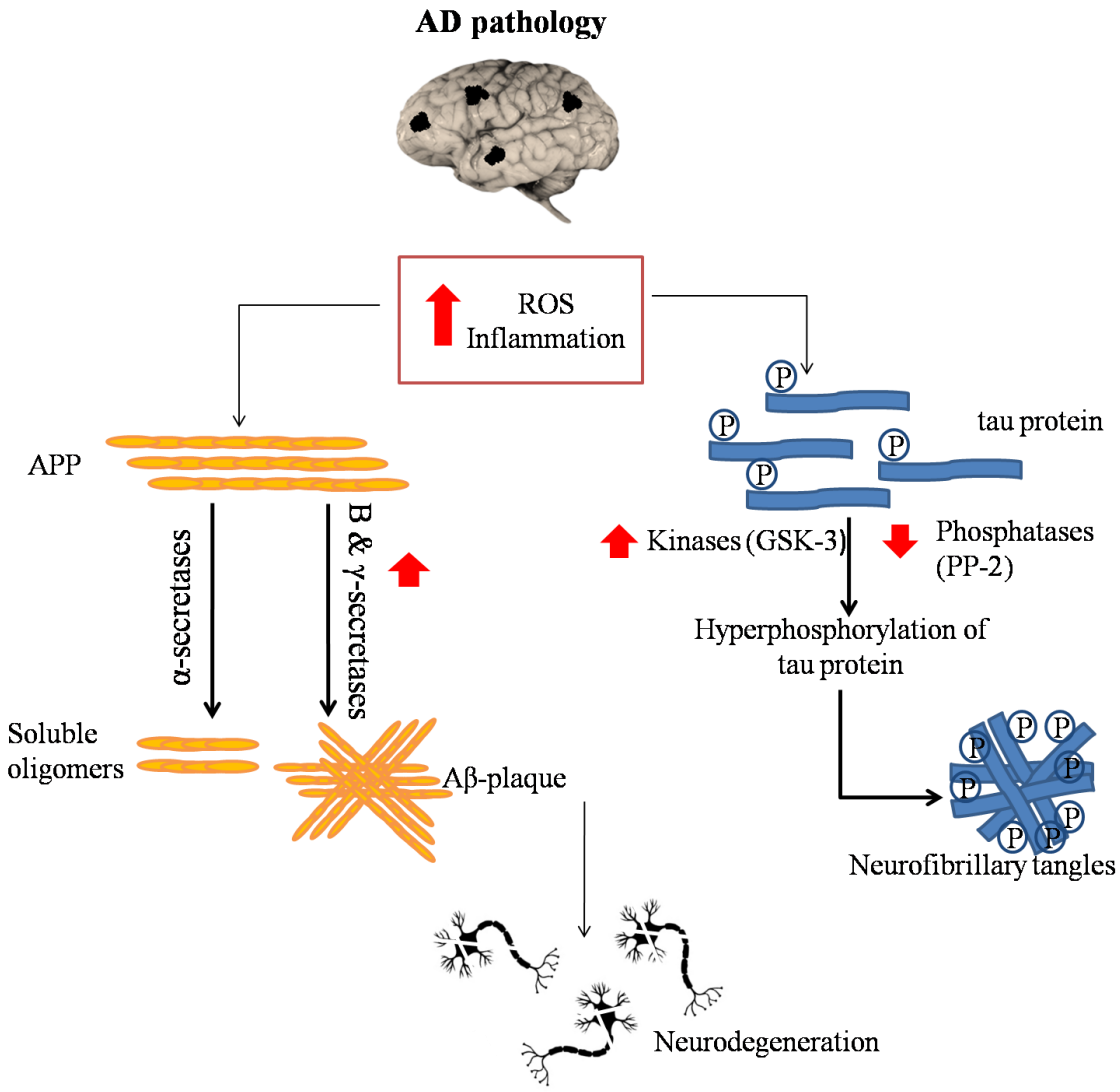

Figure 2.1 Pathophysiology of Alzheimer's disease. An increase in oxidative stress, inflammation, amyloid plaque deposition and formation of neurofibrillary tangles leads to neurodegeneration and symptoms characteristic of AD.

The mature microbiota of an adult consists of over a 1000 bacterial species and 7000 different strains (Toole, 2012). Two bacterial phylo-types that is Firmicutes and Bacteroidetes contribute majorly to the adult gut microbiome while Actinobacteria, Fusobacteria, Proteobacteriaand Verrucomicrobiaphyla are present in lower abundance. Microbiome composition varies significantly between individuals and is thought to be determined by genetics of host (Grenham et al., 2012).

Previously, gut colonization was considered a postnatal event and the intestines of fetus were thought to be sterile in utero. New evidence has emerged that suggests some bacteria and bacterial by-products may be transmitted from mother to fetus through blood in umbilical cord and the amniotic fluid (Rautava et al., 2016). True gut colonization occurs during birth when a newborn baby is exposed to environmental flora. Colonization is affected by mode of delivery that is caesarean section or a natural birth and the health status of mother (Forsgren et al., 2017; Houghteling & Walker, 2015)). Other factors affect colonization during infancy such as early exposure to antibiotics, infections, premature birth, hospitalization, breastfeeding and diet. The microbiota matures till childhood and then becomes relatively stable in composition (Fassarella et al., 2012; Walker et al., 2017).

Gut microbiota partakes in several functions in host body. It promotes gut health by maintaining intestinal barrier, producing mucous and regeneration of intestinal epithelial cells (Nell & Josenhans, 2010). It develops and matures immune system by stimulating the innate immunity and acquired immunity by invoking local and systemic responses leading to maturity of intestinal linked lymphoid tissue (Jandhyala et al., 2015). It also supports synthesis of vitamin K, hormones such as gastrin and metabolism and uptake of

certain nutrients (Ellis et al., 2021; Liew &Mohd-Redzwan, 2018). Finally, it also plays a role in detoxification of toxins and poisons (Guerre, 2020; Wang & Wang, 2016).

2.4 GBA bi-directional communication routes

The GBA is considered to be bi-directional since the gut microbiota influences the function of CNS (bottoms-up approach) and the CNS influences the composition of gut microbiota (top-down approach). The GBA has several routes of communication which include the neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis, the BBB and intestinal mucosal barrier, the vagus nerve and synthesis of neuromodulators (Tillish, 2014).

2.4.1 Hypothalamic-pituitary-adrenal axis

The gut microbiota supports the hypothalamic-pituitary-adrenal (HPA) axis' development. In several studies, germ free (GF) mice have been observed to respond to mild restraint stress with an elevated stress response. A significantly increased production of stress hormones is observed in GF mice in response to mild restraint stress. This abnormal response can be ameliorated by fecal microbial transplantation of *Bifidobacterium infantis* into host gut indicating its importance for normal postnatal development of stress response (Sudo et al., 2004). GF male Balb/c mice in comparison to specific pathogen-free (SPF) mice exhibited higher plasma corticosterone response to acute restraint test. GF mice exhibited higher expression of Crhr1 which is initiates stress response (Vagnerová et al., 2019). Microbiota modulates HPA axis and emotional

response in social conflicts using GF mice and SPF mice, as shown by another study (Vodička et al., 2018).

Early life stress may change gut microbiota composition through the release of cortisol by HPA axis and alternations in levels of circulating proinflammatory cytokines. Maternal separation in rhesus monkey aged 6 to 9 months resulted in a significant decrease in fecal lactobacilli, on the third day after the separation was initiated (Bailey & Coe, 1999).C3H/HeN mice exhibited sex-specific gut dysbiosis in response to early life adversity. Female mice exhibited a dysbiosis in *Lactobacillus* and *Mucispirillum* genera whereas male mice had dysbiosis in phyla Firmicutes and *lactobacillus, Bacteroides* and *Alloprevotella*genera (Rincel et al., 2019).

Chronic stress affects the integrity of intestinal mucosal barrier leading to a leaky barrier and translocation of bacterial cell wall components such as lipopolysaccharides (LPS). LPS affects blood brain barrier integrity leading to a pro-inflammatory cytokine response (Köhler et al., 2016). Translocation of bacterial components has been indicated in stress linked neuropsychiatric diseases such as depression. Barrier leakiness due to stress linked HPA response can be modulated by *Lactobacillus farciminis*, a potential probiotic (Ait-Belgnaoui et al., 2006). A microbiota cocktail of human origin containing 5 different strains of *Enterococcus* and *Lactobacillus* respectively prevented leaky gut, gut dysbiosis and decline in cognitive functioning of aging mice being fed diets that werehigh in fat (Ahmadi et al., 2020)

2.4.2 Vagus nerve

The vagus nerve is the 10th cranial nerve and originates from the brain and extends to the mucosal and muscular gut layers. It plays several roles including regulation of peristalsis in gut, satiety and GI secretion (Breit at al., 2018). 80% of the vagus nerve is composed of afferent sensory pathways that relay information from the gut to brain regarding nutrition, immunity and composition of microbiome (Borre et al., 2014). The efferent sensory pathway, on the other hand, stimulates the CNS to induce a systemic anti-inflammatory. The CNS responds by diminishing release of pro-inflammatory cytokines which prevents pyosepticemia caused by microbes in the gut (Forsythe et al., 2014). Therefore, the vagus nerve serves as an important interface for bi-directional communication between gut and brain interactions.

2.4.3 Neuromodulators

Neuromodulators are chemical substances that affect the excitatory or inhibitory responses of receptors in conjunction with neurotransmitters. Important neuromodulators synthesized by gut microbiota are γ-Aminobutyric acid (GABA), short chain fatty acids (SCFAs), Brain Derived Neurotrophic Factor (BDNF) and serotonin (Bauer et al., 2016). Gut microbiota synthesize essential SCFAs like acetate, butyrate and propionate by metabolizing dietary fibers. SCFAs serve as energy sources. They also play functional roles in host bodies. They function as histone deacetylases inhibitors and regulate mRNA expression of BDNF (Venegas et al., 2019)). SCFAs also regulate GI motility, oxygen consumption, heart rate and glucose metabolism (Kimura et al., 2011). Carbon radioisotope was used to label dietary fiber in a study that demonstrated that SCFAs

produced by the microbiome can penetrate the BBB. Acetate produced from radioisotope labeled dietary fiber accumulated in the hypothalamus after crossing the BBB and led to suppression of appetite by modulation of regulatory neuropeptides (Frost et al., 2014).

GABA is an inhibitory neurotransmitter of the CNS. It is primarily synthesized within the brain by glutamine-glutamate-GABA cycle. It is also synthesized within the GI tract and enteric nervous system (Spiering, 2018).It is demonstrated that GF mice have lower levels of GABA within gut, exhibiting potential GABA production by gut microbiome (Matsumoto et al., 2012). Strains of *Bifidobacterium* and *lactobacillus* isolated from gut synthesized GABA when grown media containing glutamate (Barret et al., 2012).

BDNF is a neurotrophin present abundantly in human cortex. It is responsible for coordinating synaptic plasticity, synaptic formation and function as well as enhancing neuroimmune responses. It regulates neuronal proliferation, differentiation and neuronal survival, ultimately modulating the ability to memorize and learn (Wu et al., 2013; Lu et al., 2013; Bauer et al., 2016). Animal studies illustrate the microbiomes role in BDNF production. Chronic exposure to alcohol consumption in mice led to dysbiosis of gut microbiota in *Turicibacterspp, Allobaculum spp* and *Adlerdruetzia spp* resulting in altered HPA axis activity resulting in anxiety-like and depressive behaviors and a decrease BDNF expression (Xu et al., 2019).

Serotonin is a monoamine neurotransmitter which regulates behavior, cardiovascular functions, peristalsis and secretion in GIT and respiration. 95% of serotonin found in the body is situated within the gut. Gut biosynthesis of serotonin is independent of the biosynthesis in brain (Spohn & Mawe, 2017). The biosynthesis of serotonin is dependent on microbiota-host interactions as microbiota increases expression of tryptophan

hydroxylase in enterochromaffin cells where 90% of gut serotonin is localized. This enzyme catalyzes serotonin biosynthesis's rate limiting step (Yano et al., 2015). Changes in serotonin expression are implicated in many neuropsychiatric diseases, particularly depression.

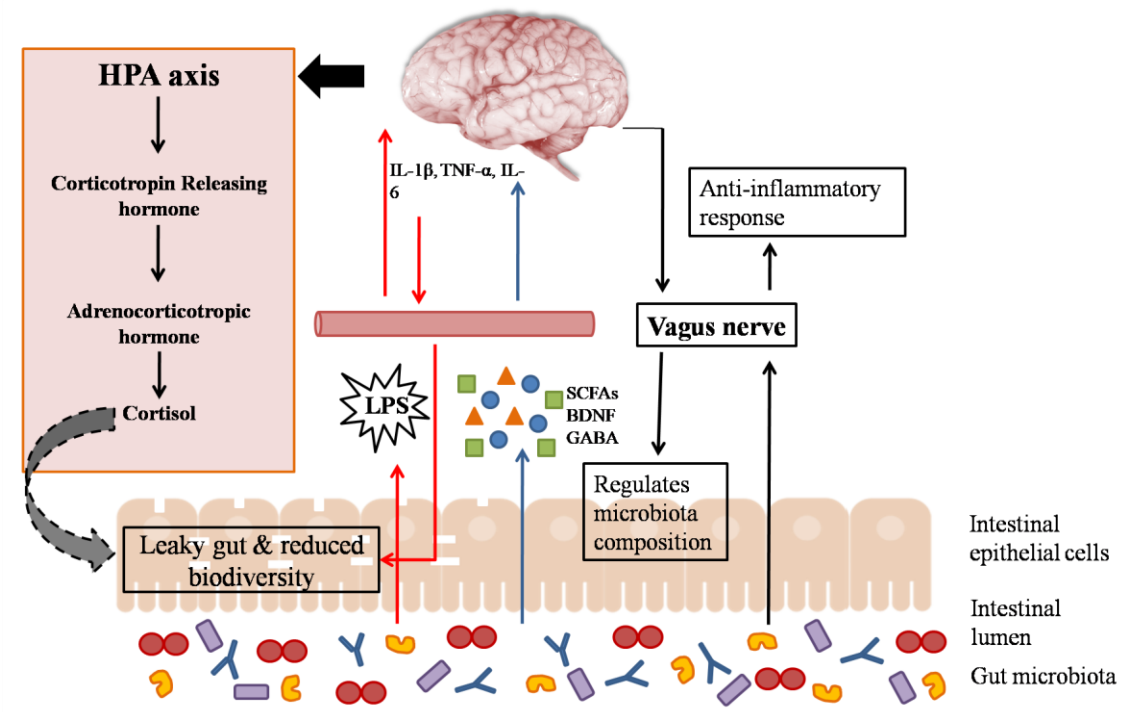

Figure 2.2 Bi-directional pathway of Gut Microbiota-Brain axis. The gut microbiota and brain interact through the HPA axis, neuromodulators and vagus nerve.

2.5 AD and Gut Microbiota-Brain axis

Although gut microbiota role in its host health has been previously established, data about changes in gut microbiota composition as consequence of AD remains scarce. Changes in gut microbiome composition are known as dysbiosis.

A Chinese cohort identified the differences in gut microbial colonies of AD patients using 16s ribosomal RNA miseq sequencing technique. The study revealed that the proportions of three key phylas were altered significantly in gut microbiota of AD patients. The relative abundance of firmicutes was significantly lower ($p = 0.008$) whereas that of proteobacteria was significantly increased ($p = 0.024$). Bacteroidetes were also observed to be lower in AD patients. Firmicutes are important SCFA producing bacteria. SCFAs play an essential role in protecting BBB and maintaining mucosal barrier permeability. A reduction in circulating SCFAs leads to BBB damage and leaky gut. Proteobacteria are proinflammatory bacteria that contribute to LPS accumulation and secrete cytokines. *Clostridiaceae* and *ruminococcaceae* were present in lower abundance in AD patients. Their lower abundance has been linked to insulin resistance. Insulin resistance is an important risk factor for AD (Liu et al., 2019).

Furthermore Cattaneo et al., 2017 reported a differential expression of anti-inflammatory and pro-inflammatory cytokines. Proinflammatory cytokine (CXCL2, IL6 and IL-1β) expression was increased and anti-inflammatory cytokine (IL-10) expression was reduced in peripheral blood of AD patients. Abundance of *Eschericha* and *Shigella* in gut were positively correlated to the amount of proinflammatory cytokines (Cattaneo et al., 2017). Another study also reported dysbiosis in AD patients including a relative decrease in abundance of firmicutes and *Bifidobacterium* and increase in bateroidetes (Vogt et al., 2017). This evidence dictates that influencing diversity and composition of microbiome through various means may serve as a potential treatment route to delay the onset and progression of disease.

2.6 Probiotics

Probiotics are living microbes which benefit host health by colonization of the gut when they are ingested in sufficient quantities (Hill et al., 2014). Ingestion of probiotics leads to beneficial effects such as improvement in gut health and modulation of gut mucosa permeability leading to prevention of bowel diseases. They also display antihypertensive, antidiabetic and antihypercholesterolemic effects and improve development and functioning of immunesystem (Alok et al., 2017).

The health benefits conferred to host are dependent on species of probiotics ingested. The most beneficial strains of probiotics are thought to be *Lactobacillus* and *Bifidobacterium*. *L. acidophilus,L. rhamnosus, L. plantarum, B. longum, B. lactis* and *B. bifidum* are the most thoroughly investigated representative species (Liu et al., 2015).

The Italian Ministry of Health (IMH) proposes consumption of a minimum of $1x10^9$ CFUs per day of viable probiotic cells for conferring of health benefits (Ohr, 2010). Probiotics are a relatively low cost, well tolerated and widely available intervention (Den et al., 2020).

Since scientific evidence dictates dysbiosis in gut of AD patients and probiotics have the ability to modulate gut microbiome composition they may serve as a novel approach for treatment of AD.

2.6.1 *Lactobacillus rhamnosus* GG

Lactobacillus rhamnosus GG is a gram-positive rod-shaped facultative anaerobe found ubiquitously in genitourinary tract of females and GIT. They are often referred to as LGG® by commercial manufacturers. The GG refers to the specific strain of bacteria. LGG® belongs to *rhamnosus* species, *Lactobacillus* genus and the phyla Firmicutes (Albarillo et al., 2020).

Over 250 clinical trials have been conducted using LGG® with subjects ranging from premature newborns to adults and for the treatment of a host of different diseases (Dronkers et al., 2020). Several studies indicate that LGG® reaches the gut alive and has the ability to colonize the gut (Petschow et al., 2005). Moreover, it is very well tolerated and rarely has any significant side effects (Capurso, 2019).

Dysbiosis of gut may lead to lower bioavailability of GABA leaving the CNS vulnerable to excitotoxicity. *Lactobacillus* species convert monosodium glutamate into GABA. A study reported that *Lactobacillus rhamnosus* modulates emotional behavior by regulating GABA receptor expression (Bravo et al., 2011). Another study reported anti-inflammatory effect by prevention of induction of TNF-α and IL-8 in human colon epithelial cells (Lee et al., 2012). Therefore, LGG® is a good candidate for treatment of AD.

2.6.2 *Bifidobacterium*BB-12®

*Bifidobacterium*BB-12® is a non-motile and non-spore anaerobic forming microbe (Reuter, 2001). It is a gram-positive, rod-shaped bacteria that produces lactic acid.

*Bifidobacterium*BB-12®was first classified as *Bifidobacterium lactis*. It was later reclassified as *Bifidobacterium animalis* subspecies *lactis*. It belongs to the pyla Actinobacteria. It is a microbe that commonly inhabits the GIT (Jungersen et al., 2014).

Over 130 clinical trials have been conducted using BB-12®as a therapeutic agent. It is excellent at colonizing the GIT, this quality can be attributed largely to its tolerance for gut acid and bile. BB-12®has high survival rates and tolerance for low pH ranging from pH 2-5. It also performs well in mucus adhesion studies (Harata et al., 2021). A study reported that BB-12®adhered to poly-carbonate well plates with or without mucin (Laparra & Sanz, 2009). Acid and bile tolerance along with mucus adhesion are the most important considerations for selection of probiotics.

BB-12®inhibits colonization of gut by pathogens through various mechanisms such as activation of host immune system, competition for adherence sites on intestinal epithelial cells, toxin removal and production of inhibitory substances like bacteriocins, H_2O_2 and organic acids (Jungersen et al., 2014).BB-12®inhibits pathogenic bacteria including *Escherichacoli, Shigella flexneri, Enterococcus faecalis*in vitro. *Eshcericha* and *Shigella* dysbiosis in gut has been implicated in AD. Therefore, BB-12®is a good potential treatment for AD (Martin et al., 2009).

A

B

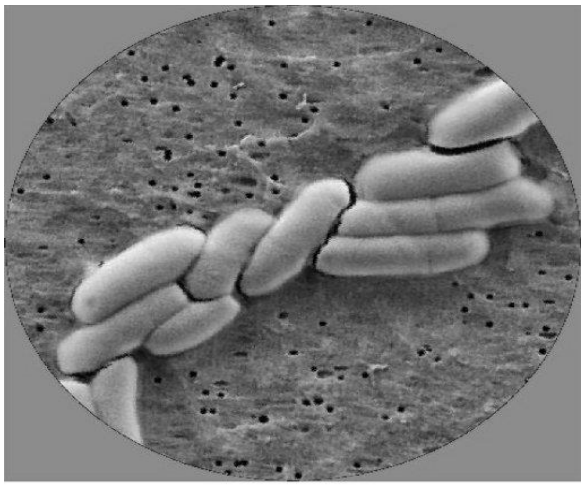

Figure 2.3 SEM image of (A) *Lactobacillus rhamnosus and* (B) *Bifidobacterium* BB12(adopted from Garcia-Hernandez et al., 2015 and Bozkurt et al., 2019, respectively).

MATERIALS AND METHODS

3.1 Chemical and reagents

Aluminum Chloride hexahydrate ($AlCl_3.6H_2O$) was purchased from Scharlau (Product catalogue no. AL0770). Lyophilized pre-packaged probiotic formulation of *Lactobacillus rhamanosus* GG *and Bifidobacterium lactis* BB-12 manufactured by Chr. Hansen, sold under brand name Imutec; and donepezil (Donecept®) manufactured by ATCO were brought from local pharmacies in Islamabad, Pakistan. Chemicals required for electrophoresis and gene expression analysis were obtained from Sigma Aldrich.

3.2 Animals

Balb/c mice of age 6-8 weeks and male gender, weighing 20-30g were bred and housed in LAH of ASAB, NUST. Mice were housed at room temperature (25±2°C) in a 12-hour dark and light cycle in standard cages. A standard diet and *ad libitum* access to distilled water was provided.

3.3 Ethical statement

The experiments were performed in consonance with the resolutions of World Medical Association, declaration of Helsinki which states that all those who produce and use animals for research purposes are responsible for their wellbeing. Institutional Review Board (IRB) of ASAB, NUST under ethical code IRB-09-2022-ASAB-01/01 approved the present study.

3.4 Development of AD mouse model

For screening of probiotic potential to ameliorate the progression and severity of AD an $AlCl_3$ neurotoxicity model though oral administration was developed. To induce AD, $AlCl_3$ mediated neurotoxicity model was used. Animals were randomly distributed into 6 groups such that each group comprised of 8 subjects (n=8). Total number of mice is n_t=48. Groups 1, 2 and 3 served as controls and were provided with distilled water and groups 4, 5 and 6 were orally administered $AlCl_3$ (300mg/kg) for 15 days according to pre-established protocols (Amber et al., 2018).

Table 3.1 Experimental groups for $AlCl_3$ toxicity model

Sr. No.	Experimental groups		Treatment	
		(n)	15 days	28 days (oral administration)
1	Control	8	Distilled H_2O	Distilled H_2O
2	Donepezil	8	Distilled H_2O	Donepezil 15mg/kg
3	Probiotics	8	Distilled H_2O	Probiotic $1*10^9$ CFUs
4	$AlCl_3$	8	300mg/kg $AlCl_3$	Distilled H_2O
5	$AlCl_3$ + Donepezil	8	300mg/kg $AlCl_3$	Donepezil 15mg/kg
6	$AlCl_3$ + Probiotics	8	300mg/kg $AlCl_3$	Probiotic $1*10^9$ CFUs

3.5 Experimental design

A 15-day long protocol was designed to generate AD mouse model (n=48) by oral administration of $AlCl_3$ (300mg/kg). This was followed by 28 days of oral administration of Donepezil (15mg/kg) (Ahmed et al., 2017; Kwon et al., 2014) and probiotics ($1x10^9$ CFU). Behavior tests for anxiety, spatial learning and memory, reference memory and recognition memory were carried out for the next 14 days followed by histological

analysis. Genomic analysis was performed through real time quantitative PCR (Figure 3.1 and 3.2).

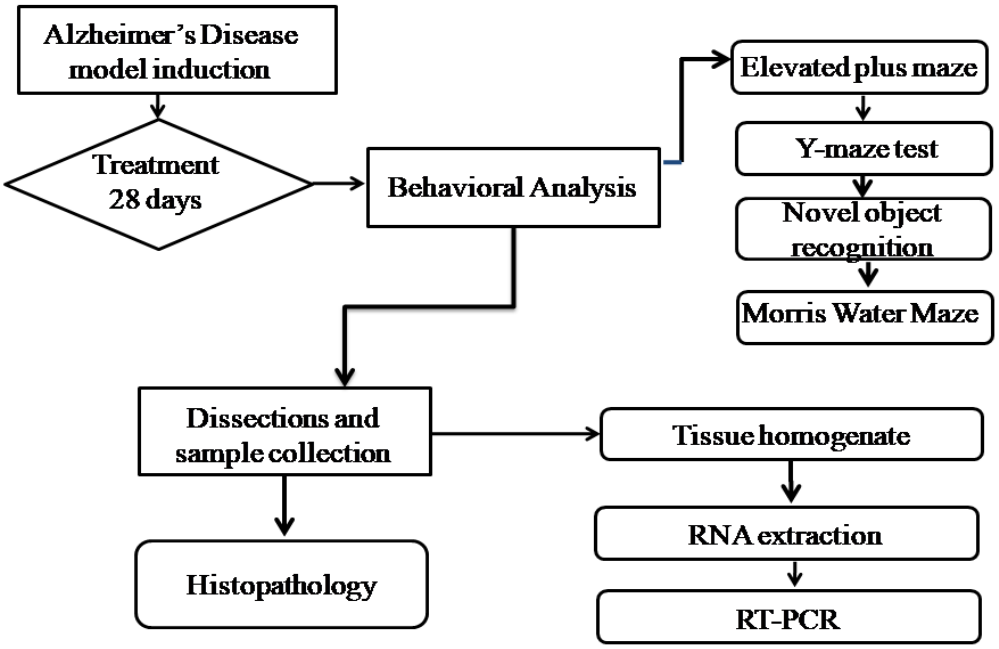

Figure 3.1 Experimental design

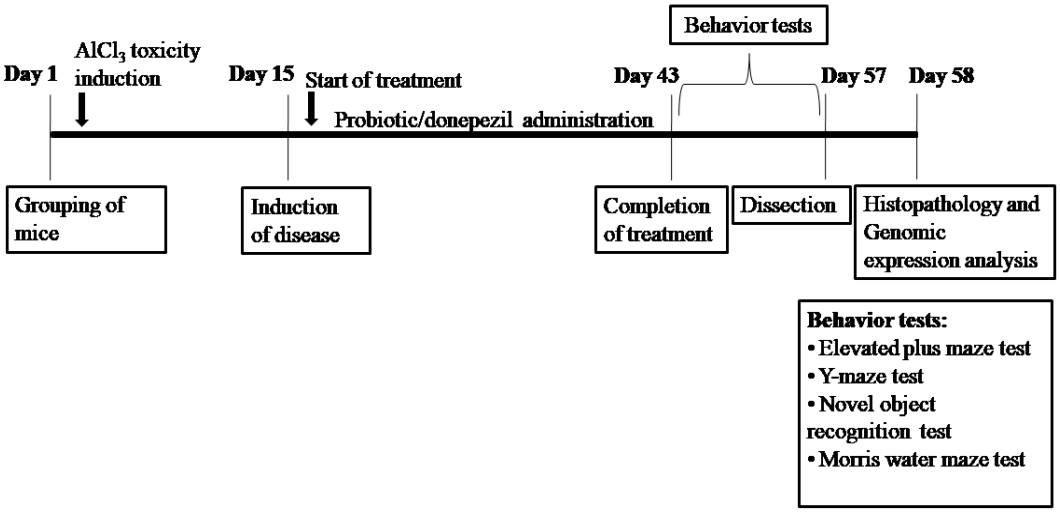

Figure 3.2 Study Design. Induction of AD via $AlCl_3$-inducedtoxicity for 15 days followed by treatment with donepezil and probiotics for 28 days, behavioral analysis and sacrifice of animals for genomic and histological analysis.

3.6 Behavioral studies

Four behavioral tests were conducted to analyze effect of probiotic treatment on behaviors; Elevated Plus Maze (EPM) test, spontaneous Y-maze test, Novel Object Recognition (NOR) test and Morris Water Maze (MWM) test. Behavioral tests were performed according to behavioral battery in which each subsequent test has increasing invasiveness with a one-day recovery period between each subsequent test to reduce carryover effects from handling and testing procedures (Puścian et al., 2014; Wolf et al., 2016). Male gender mice were selected to minimize estrous cycle's effects on anxiety and cognitive behaviors displayed by females (ter Horst et al., 2012).

3.6.1 Elevated plus maze test

EPM test is a measure of anxiolytic behavior in rodents. Rodents are averse to open spaces and heights and this test measures the tendency to explore the open spaces despite aversion. It was conducted in accordance with pre-established protocol by (Arendash et al., 2004). The apparatus comprised of 4 arms of which 2 were enclosed alleys and 2 were open alleys. The apparatus was made up of opaque iron alloy, was elevated 75.5cm from the ground and had 30 x 5cm arm each. Each mouse underwent a single 5-minute trial by being placed at the intersection of the maze facing away from experimenter and towards any one of the two enclosed arms. A video was recorded for behavior analysis focusing on (a) the total number of entrances into the open arms of EPM and (b) cumulative time spent in both of the open arms. 70% ethanol spray was used to clean apparatus between subsequent trials to prevent altering of behavior due to olfactory cues.

3.6.2 Y-maze test

Y-maze task was conducted to assess spatial learning and memory. The protocol was adapted from Conrad et al., 1996. The Y-maze is composed of three arms each at a 120° angle from the adjacent one. The arms were categorized as start arm, other arm and novel arm. The start arm served as the location where mouse was initially placed and allowed to explore along with the other arm during the habituation phase. The novel arm was kept blocked using a thick wooden plank during the first trial and unblocked during the second one. The maze was kept in an undisturbed quite room at 25 ± 2 °C temperature. Different visual cues were hung in all three arms such that they were in line of sight of the test subject.

The first trial lasted 10 minutes while the test subject explored the Y-maze. After an inter-trial interval of 10 minutes, the subject was placed back into the maze to explore for another 5 minutes with novel arm unblocked. The data was analyzed as percentage (%) spontaneous alternations.

$$\%\ spontaneous\ alternations = \frac{number\ of\ spontaeneous\ alternations}{total\ number\ of\ alternations - 2} \times 100$$

3.6.3 Novel Object Recognition test

Recognition memory is tested using novel object recognition (Farhat et al., 2017). It consists of two test trials of 10-minutes each with an inter-trial interval of 20 minutes. The subjects were allowed to acclimatize to a square wooden box of (42 cm × 42 cm × 42 cm) for 5-minutes 24 hours prior to familiarization and test session.

In the familiarization session two similar objects were placed into the box being used to carry out NOR test and mouse was allowed to interact freely with the objects. After termination of familiarization session one of the two familiar objects was removed. In place of the removed object a new object was placed. This new object was termed as the novel object. After completion of inter-trial interval mouse was allowed to explore and interact with both objects. Sniffing or physically touching the object was considered as exploration. The following formula was used to calculate discrimination index:

$$\text{Discrimination index} = \frac{time\ spent\ with\ novel\ object}{time\ spent\ with\ novel\ object + time\ spent\ with\ familiar\ object}$$

3.6.4 Morris water maze test

MWM is an important test for AD model that tests hippocampal-dependent learning and long-term spatial memory. It consists of a five-day acquisition phase and then a single day probe trial. The MWM consists of a circular metal pool. The pool was divided into four quadrants (east, west, north, south) with visual cues placed in each quadrant. The MWM contains a transparent platform and is filled with water (22℃ ±2 ºC). The transparent platform was placed in the North-West quadrant.

The acquisition phase consisted of five days. On each day five trials were conducted with inter-trial interval of 10 minutes per mouse. The mice were placed gently into the pool from different directions. The directions in which they were released were according to the arrangement in Table 3. The cut off time was 90 seconds. If the mouse failed to reach the platform within 90 seconds it was guided to it and held in place for 20 seconds. In the

case that mouse was able to find the platform before cut-off time; the mouse was allowed to sit on the platform for an additional 5 seconds before being removed.

To perform probe trial on the 6thday the platform was removed from the maze. The mice were given 90 seconds to explore the maze and a video was recorded. Four parameters were calculated (Bromley-Brits et al., 2011):

- Escape latency over 5 days during acquisition phase
- Number of crossings over platform during probe trial
- Number of entries into target quadrant during probe trial
- Time spent in target quadrant during probe trial

Table 3.2 Trial directions for MWM test

No. of Days	**Release Directions**				
	Trial 1	***Trial 2***	***Trial 3***	***Trial 4***	***Trial 5***
01	South	West	East	North	South
02	West	South	North	West	East
03	East	West	South	North	East
04	South	North	East	West	South
05	North	South	West	East	North
06	West: the direction of release.				

3.6.5 Statistical analysis

The software was analyzed using Graphpad prism version 8.0.1 to apply tests of One-way analysis of variance (ANOVA) or two-way ANOVA to dataset to determine whether statistical significance exists. Bonferroni's multiple comparison test was also applied to determine group to group differences. Mean ± SEM was used to present error bars.

3.7 Brain Dissections

Chloroform was used to anesthetize mice prior to euthanasia by cervical dissection. Using forceps, the head was gently stretched forward and surgical scissors were used to make a cut posterior to the ears. A small incision was made from the base of the skull at the parietal bone. Both sides of the parietal bone were tilted and broken off to reveal the brain. Frontal bone was also cut to gently remove the brain. The brain was washed in PBS to clear off excess blood and placed on a cold metal tray to section.

The first step was to remove cerebellum and olfactory bulb using a sharp scalpel. The brain was placed with ventral side facing up and curved forceps were used to lightly pull apart the two halves off the cerebrum. After making an opening along the midline the forceps were moved anticlockwise first to open left hippocampus and then clockwise to reveal right hippocampus at an angle of 30-40°. The hippocampus and the cerebrum were removed and stored separately in microcentrifuge tubes. The samples were frozen at -80°C until further use.

3.8 Histological examination

3.8.1 Tissue perfusion and slide preparation

For histological analysis heart perfusion was performed according to Gage et al., 2012. Briefly, the whole brain was removed from skull and washed with PBS before being transferred to 4% paraformaldehyde (PFA) solution for 24 hours and was swirled occasionally. The brain tissue was then dehydrated with 70%, 95% and 100% isopropanol consecutively for 1 hour each. Before tissue permeation the tissue was incubated in xylene for 4 hours. Molten paraffin was poured over tissue sample for paraffin embedding. The temperature was maintained at 60°C. The embedded sample was left to solidify at 4°C prior to cutting.

3.8.2 Congo red staining

Tissue sections were de-paraffinized and rehydrated before staining with Congo red stain for 20 minutes using a solution of 0.5 mL 1% NaOH and 49.5 mL Congo Red. The sections were then washed twice using double distilled water (ddH_2O) and dipped in an alkaline alcohol solution for 2 minutes. Hematoxylin was used to counterstain the sample for 30 seconds and washed for 6 minutes with 70% isopropryl alcohol. The final step involved the washing of the samples with ddH_2O. After air drying the slides for 1hour, the cover slips were placed. The images were visualized at 4X, 10X and 40X magnification using inverted light microscope. Images were acquired and analyzed using OPTIKA Lite Software Version 2.11. Congo red stains Aβ aggregates as red-pink.

3.9 Gene expression analysis

3.9.1 RNA extraction

Tri-reagent protocol was followed for RNA extraction. Tissue samples were homogenized in 1ml Trizol using Ultrasonic Processor UP400S (Hielscher Ultrasound Technology) and incubated for 15 minutes at 4°C. Each sample was shaken for 15 seconds after adding Chloroform (200µl), Samples were incubated for 10 minutes at 4°C. Refrigerated centrifuge was set to 12,000 rpm at 4°C for and sample was centrifuged for 15 minutes. Colorless upper aqueous phase obtained after centrifugation was removed gently without disrupting or mixing the layers and added to new autoclaved tubes.

Isopropyl alcohol (500µl) was added to the samples. They were incubated for 10 minutes. Centrifugation was performed at 12,000 rpm for another 10 minutes to precipitate RNA. The RNA forms a pellet on side or bottom of microcentrifuge tube. After centrifugation, the supernatant was collected and discarded. Chilled absolute ethanol (1000µl) was used to wash pellet. A final centrifugation step was conducted at 7500 rpm for 5 minutes at 4°C. The ethanol was removed gently after washing to avoid dislodging of pellet.30µl DEPC water was added to pellet after air drying it. Until further use, it was stored at -80°C.

3.9.2 Quality of RNA

To determine the quality extracted RNA, electrophoresis was conducted 1% agarose gel. RNA bands were visualized on ChemiDoc (ChemiDoc™ MP System #1708280, BioRad, USA). RNA concentration in each sample was determined by nanodrop method and the

ratio of A 260/280 indicated extracted RNA quality. The A 260/280 ratio ranged between 1.8-2.0. Ratio less than 1.8 indicates presence of contaminants.

3.9.3 Reverse Transcription for cDNA synthesis

RNA was quantified by use of Biophotometer plus (Eppendorf, Germany). 3µgRNA was utilized in each reaction mixture to transcribe RNA into cDNA. The reaction mixture consists of 2µl of 10mM dNTP's, 4µl of 5X RT buffer, 1µl of 10mM oligodT (heated at 55°C for 5 minutes) and 1µl of Revert AID enzyme. Total reaction volume was brought up to 25µl by adding PCR water to reaction mixture. Thermocycling conditions for cDNA synthesis indicated in figure 3.

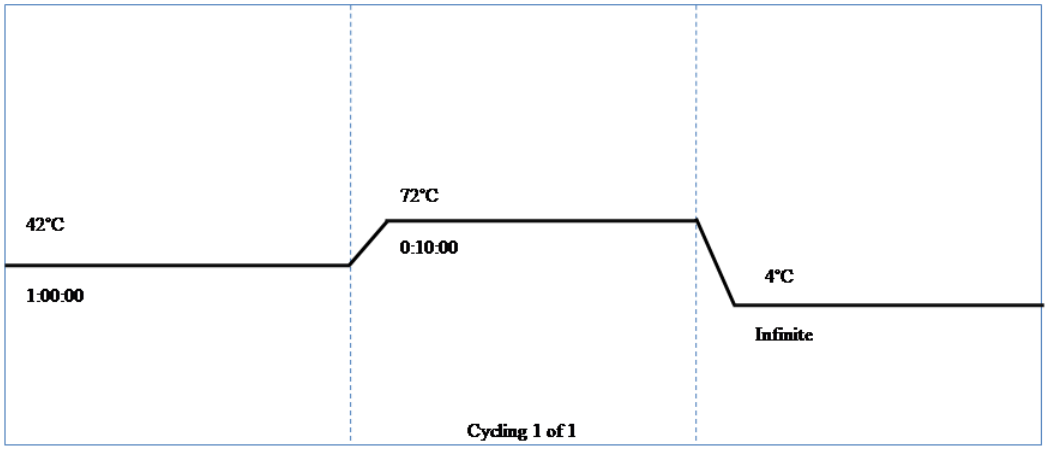

Figure 3.3Thermocycling profile for cDNA synthesis

3.9.4 Gene expression analysis

qPCR was performed using SYBR Green fluorescent dye reaction mixture. It contains 1µl of forward and reverse primers, 4µl of SYBR Green dye, 1µl of cDNA template. The volume was brought up to 20µl by adding Nuclease free water. Each sample was tested in duplicates and normalized against β-actin. Agarose gel, disassociation curves and amplification plots were used to verify the quality of RNA.

Thermocycling conditions for gene expression analysis were an initial pre-incubation step at 50°C for 2 minutes and 95°C for 10 minutes. Extension required 40 cycles of 95°C for 30 seconds, 60°C for 1 minute and 72°C for 1 minute and ended with a final disassociation step. The sequences of RT-PCR primers IL-1β, TNF-α, and β-actin are indicated below.

Table 3.3 Primer sequences for RT-PCR

Gene	Primer	Sequences
InterLeukin-1β	Forward	5'TTCAGGCAGGCAGTATCACTC-3'
	Reverse	5'-GAAGGTCCACGGGAAAGACAC-3'
Tumor Necrosis Factor-α	Forward	5'-ATGAGCACAGAAAGCATGA-3'
	Reverse	5'-ACCACGCTCTTCTGTCTACT-3'
β-actin	Forward	5'-ACCTTCAACACCCCAGCCATGTACG-3'
	Reverse	5'-CTGATCCACATCTGCTGGAAGGTGG-3'

RESULTS

4.1 Behavioral analysis

4.1.1. Effect of donepezil and LGG®& BB-12®on anxiety

Performance on EPM was used as a measure of anxiety to assess anxiolytic effects of LGG®& BB-12® in comparison to donepezil. Animals experiencing severe anxiety have a tendency to remain in closed arms, whereas animals experiencing lower levels of anxiety tend to explore the open arm more. Entries in open arm in $AlCl_3$-group (4.12±0.63) were lower ($p<0.01$) when compared to the number of entries made by control group (8.37±0.49). $AlCl_3$ + LGG®& BB-12-treated group (7.62±0.62) displayed significantly higher ($p<0.001$) entries into open arms in comparison to $AlCl_3$ + donepezil-treated group (1.87±0.61).

Donepezil-treated group (7.62±0.99) spent significantly less ($p<0.05$) amount of time in open arms in comparison to control group (16.75±1.70). LGG®& BB-12®-treated group (20.38±1.61) spent significantly more ($p<0.01$) time than donepezil-treated group (7.62±0.99). The $AlCl_3$ + LGG®& BB-12®-treated group (27.75±4.15) has significantly higher ($p<0.0001$) performance in comparison to $AlCl_3$group. The $AlCl_3$ + LGG®& BB-12®-treated group (27.75±4.15) also had significantly higher performance ($p<0.0001$) in comparison to $AlCl_3$ + donepezil-treated group (3.37±0.70). In conclusion the probiotic LGG®& BB-12® treatment displayed strong anxiolytic effect in both LGG®& BB-12® and $AlCl_3$ + LGG®& BB-12®-treated group. This indicates that this combination of probiotics has strong anxiolytic properties.

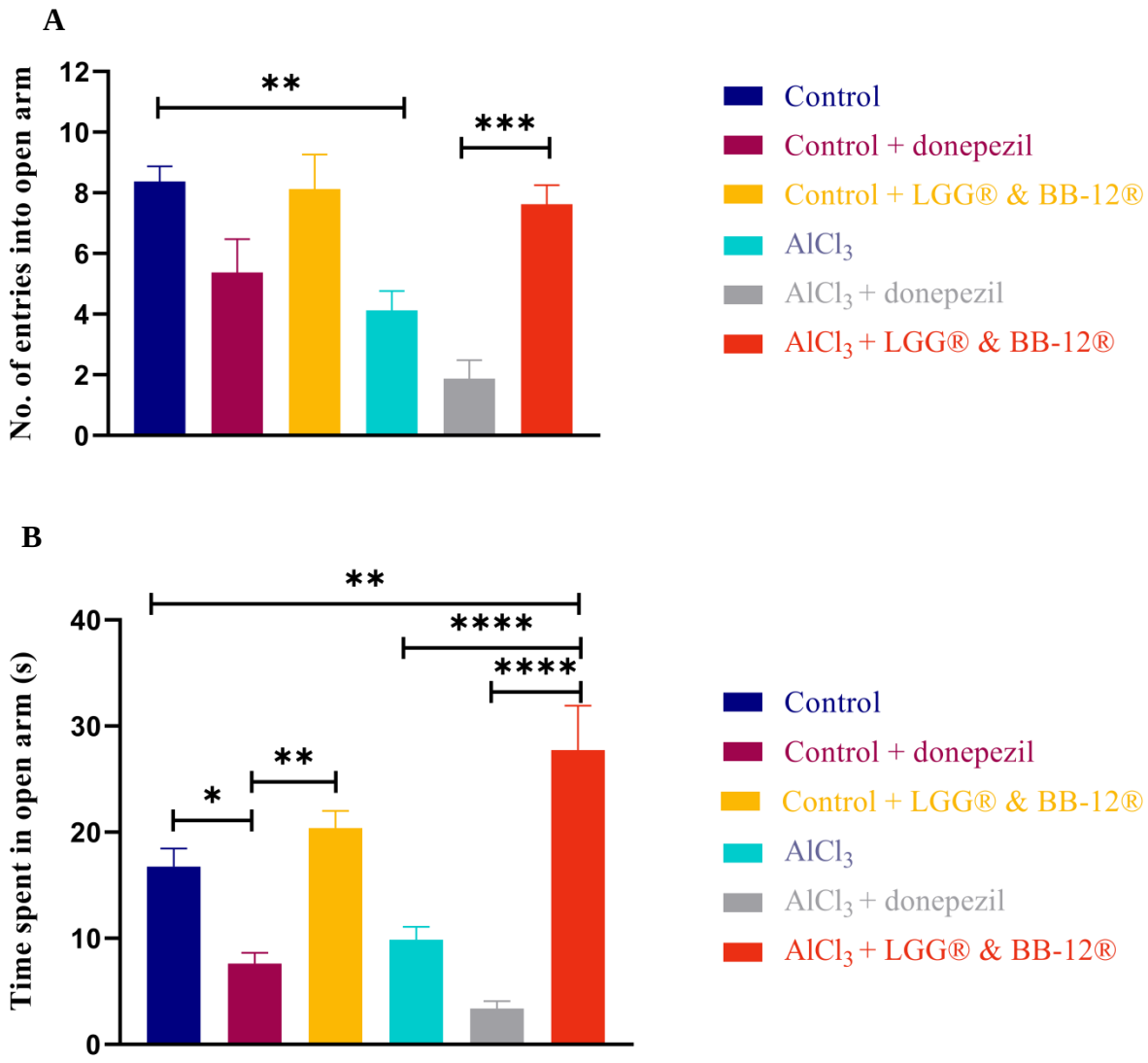

Figure 4.1 The effect of donepezil and LGG®& BB-12®on (A) Number of entries in Open arm (B) Time spent in open arm of EPM. Mean ± SEM was used to represent error bars. Statistical analysis was conducted using Graphpad Prism Version 8.0.1. "n.s=p>0.05", "*=p<0.05", "**=p<0.01", "***=p<0.001" and "****=p<0.0001"

4.1.2 Effect of donepezil and LGG®& BB-12®on spatial memory

Y-maze is a measure of spatial memory and the parameter used is percentage spontaneous alternation. Spontaneous alternations are characterized by the entry of mice into all 3 arms of the Y-maze consecutively due to their innate exploratory behavior.

The control group (77.23±1.51) had significantly better ($p<0.0001$) performance than $AlCl_3$ group (56.31±1.67). The LGG®& BB-12®-treated group (83.55±3.65) had significantly better performance ($p<0.01$) than donepezil-treated group (68.53±2.06). This indicates that LGG®& BB-12® have an effect on memory and learning. This is further corroborated by the evidence that $AlCl_3$ + LGG®& BB-12®-treated group (82.73±2.45) had significantly improved performance ($p<0.0001$) as compared to $AlCl_3$ group (56.31±1.67). $AlCl_3$ + donepezil-treated group (79.65±4.41) had significantly improved performance ($p<0.0001$) as compared to $AlCl_3$ group (56.31±1.67). The evidence suggests that the effect of oral consumption of LGG®& BB-12®in improving spatial memory in AD mouse models was comparative to the effect of the standard drug donepezil.

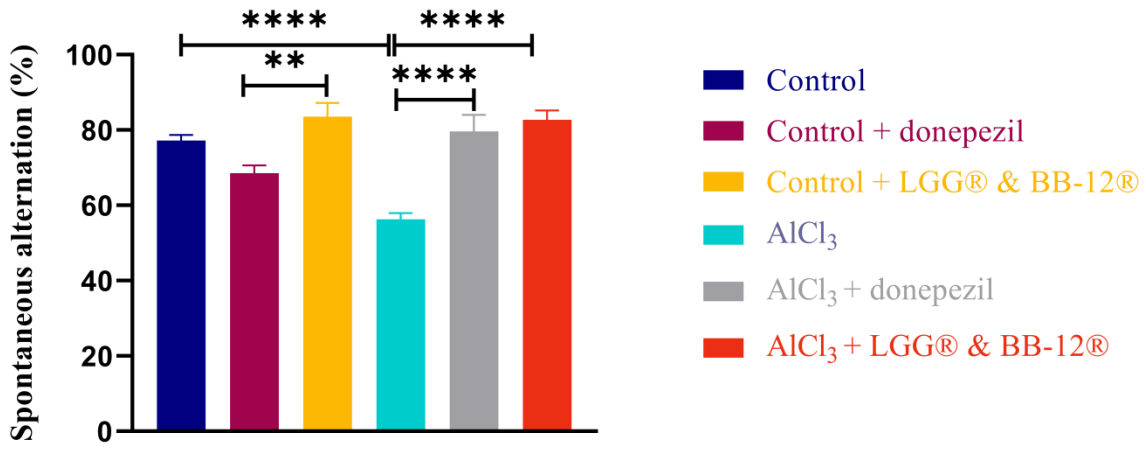

Figure 4.2 The effect of donepezil and LGG®& BB-12®on spatial learning and memory in Y-maze. Mean ± SEM was used to represent error bars. Statistical analysis was conducted using Graphpad Prism Version 8.0.1. "n.s=p>0.05", "*=p<0.05", "**=p<0.01", "***=p<0.001" and "****=p<0.0001"

4.1.3 Effect of donepezil and LGG®& BB-12®on recognition memory

Recognition memory is assessed by employing the NOR test. Rodents have a tendency to explore novel environments and objects rather than familiar ones. The recognition memory is measured in terms of DI.

In NOR test the control group (58.25±1.65) had a significantly better ($p<0.0001$) performance than $AlCl_3$ group (34.50±0.95). The $AlCl_3$ + donepezil-treated group (48.0±2.85) displayed significantly improved ($p<0.05$) performance as compared to $AlCl_3$ group (34.50±0.95). The most significant improvement ($p<0.0001$) was observed in $AlCl_3$ + LGG®& BB-12®-treated group (58.25±2.32) as compared to $AlCl_3$ group (34.50±0.95). The evidence shows that LGG®& BB-12®treatment was effective in restoring recognition memory of AD mice models.

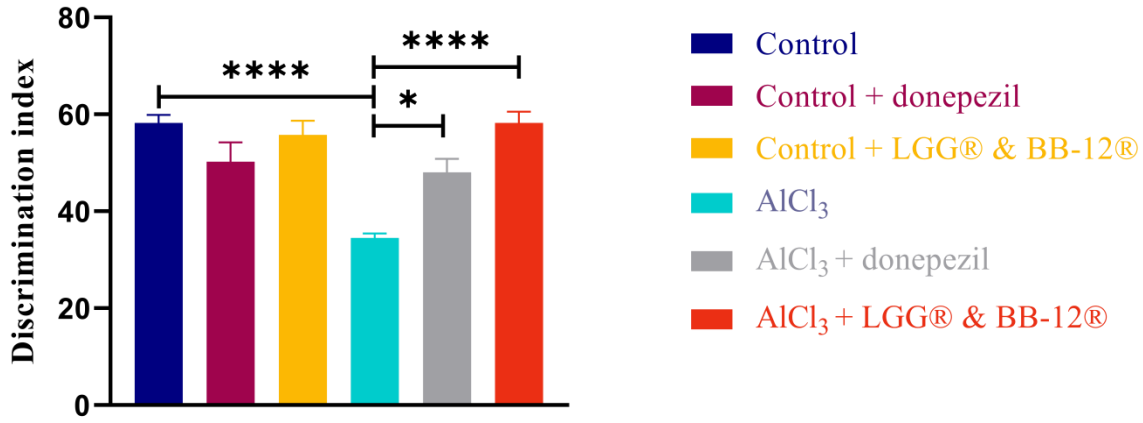

Figure 4.3 The effect of donepezil and LGG®& BB-12®on recognition memory in NOR. Mean ± SEM was used to represent error bars. Statistical analysis was conducted using Graphpad Prism Version 8.0.1. "n.s=p>0.05", "*=p<0.05", "**=p<0.01", "***=p<0.001" and "****=p<0.0001"

4.1.4 Effect of donepezil and LGG®& BB-12®on spatial learning and memory

The MWM is 6 days long and divided into training phase and probe trial. The training phase consisted of five consecutive days of trial and learning of spatial cues is measured as escape latency. The probe trial was conducted on the last day for reference memory and three parameters were measured the number of crossings over platform, amount of time spent and entries into target quadrant of maze.

On the training phases 5th day all groups displayed improvement in escape latency other than $AlCl_3$ group. $AlCl_3$ group (55.56±1.26) displayed significantly poor ($p<0.0001$) improvement in escape latency on the 5th day as compared to control group (5.85±0.63). The $AlCl_3$ + donepezil-treated group (10.53±0.63) displayed significantly improved

($p<0.0001$) escape latency as compared to $AlCl_3$ group (55.56±1.26). The $AlCl_3$ + LGG®& BB-12®-treated group (10.44±1.19) showed significant improvement ($p<0.0001$) as compared to $AlCl_3$ group (55.56±1.26). The final escape latency was 5.85s, 12.16s, 5.26s, 55.56s, 10.53s, 10.44s, 16.63s on the 5th day for control, control + donepezil group, control + LGG®& BB-12®, $AlCl_3$ group, $AlCl_3$ + donepezil-treated group and $AlCl_3$ + LGG®& BB-12®-treated group respectively.

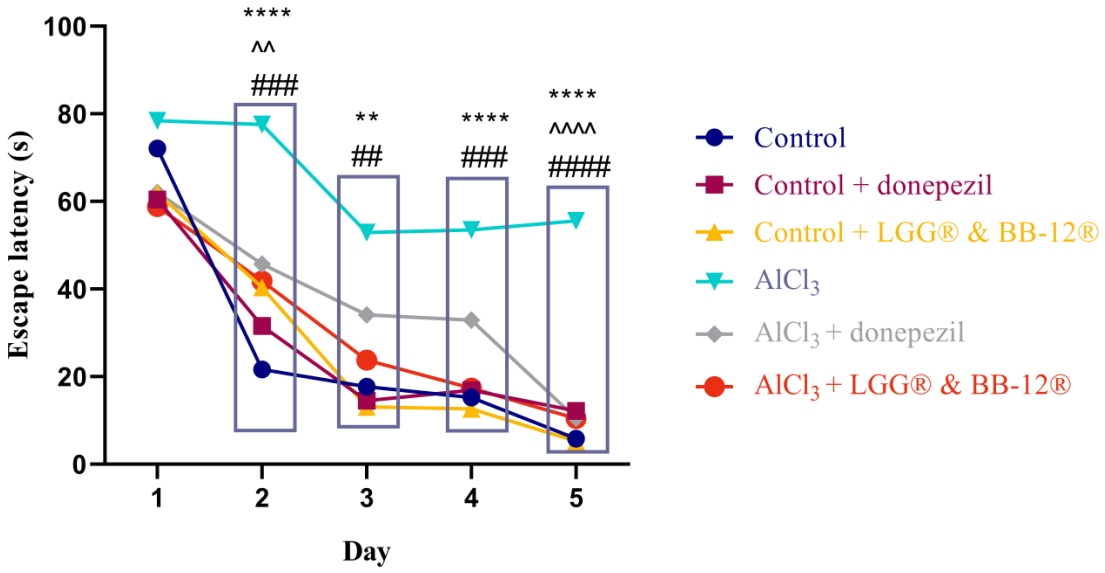

Figure 4.4 The effect of donepezil and LGG®& BB-12®on escape latency in MWM. Mean ± SEM was used to represent error bars. Statistical analysis was conducted using Graphpad Prism Version 8.0.1.Test two-way ANOVA was applied. "n.s=p>0.05", "*=p<0.05", "**=p<0.01", "***=p<0.001" and "****=p<0.0001". The symbols * , ^, # represent significance between control and $AlCl_3$group, $AlCl_3$ group and $AlCl_3$ + donepezil-treated group and $AlCl_3$ + LGG®& BB-12®-treated group respectively.

The control group (5.62±0.63) made significantly more crossings ($p<0.01$) as compared to $AlCl_3$group (1.75±0.55). The $AlCl_3$ + donepezil-treated group (4.75±0.45) had significantly higher performance ($p<0.05$) than $AlCl_3$group (1.75±0.55). The LGG®&

BB-12®-treated group (7.75±0.59) had a highly significant ($p<0.0001$) improvement as compared to $AlCl_3$group (1.75±0.55). A difference was also observed between both $AlCl_3$ treated groups, where the The$AlCl_3$ + LGG®& BB-12®-treated group (7.75±0.59) showed a significant improvement ($p<0.05$) in performance when compared to $AlCl_3$ + donepezil-treated group (4.75±0.45).

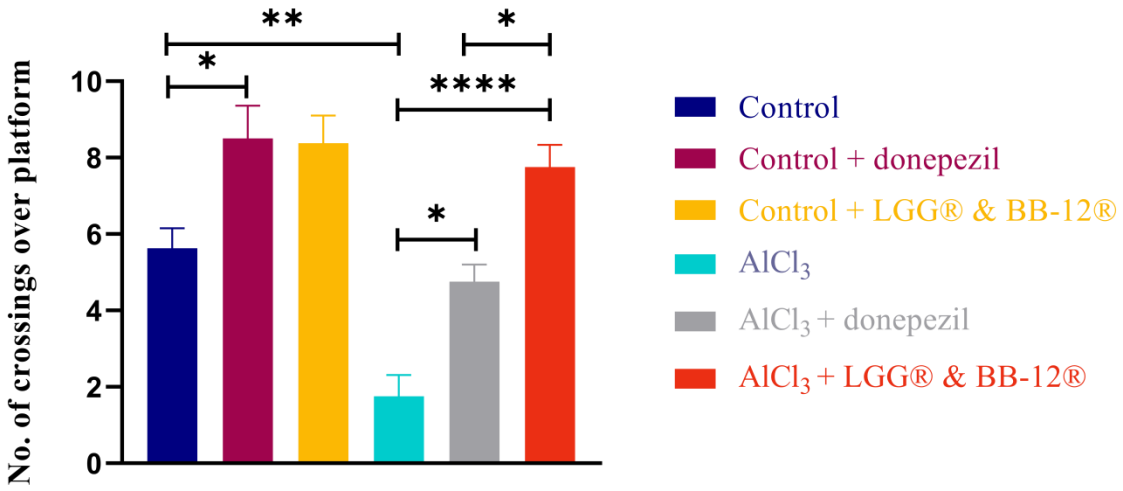

Figure 4.5 The effect of donepezil and LGG®& BB-12®on spatial memory in MWM. Mean ± SEM was used to represent error bars. Statistical analysis was conducted using Graphpad Prism Version 8.0.1 "n.s=p>0.05", "*=p<0.05", "**=p<0.01", "***=p<0.001" and "****=p<0.0001"

The number of target quadrant entries was also evaluated. The control group (10.5±0.56) showed significantly more ($p<0.0001$) entries into target quadrant as compared to $AlCl_3$ group (2.87±0.81). In the donepezil-treated group (13.5±0.82) and LGG®& BB-12®-treated group (9.87±0.61), the donepezil-treated group showed a more significant improvement ($p<0.05$). The $AlCl_3$ + donepezil-treated group (10.13±0.95) showed significant improvement ($P<0.0001$) as compared to $AlCl_3$ group (2.87±0.81). Furthermore, the $AlCl_3$ + LGG®& BB-12®-treated group (12.13±0.97) showed significant

improvement (P<0.0001) as compared to $AlCl_3$ group (2.87±0.81). This implies that the effects of LGG®& BB-12®treatment were comparable to that of donepezil. The parameter of time spent in target quadrant did not display any discernible differences.

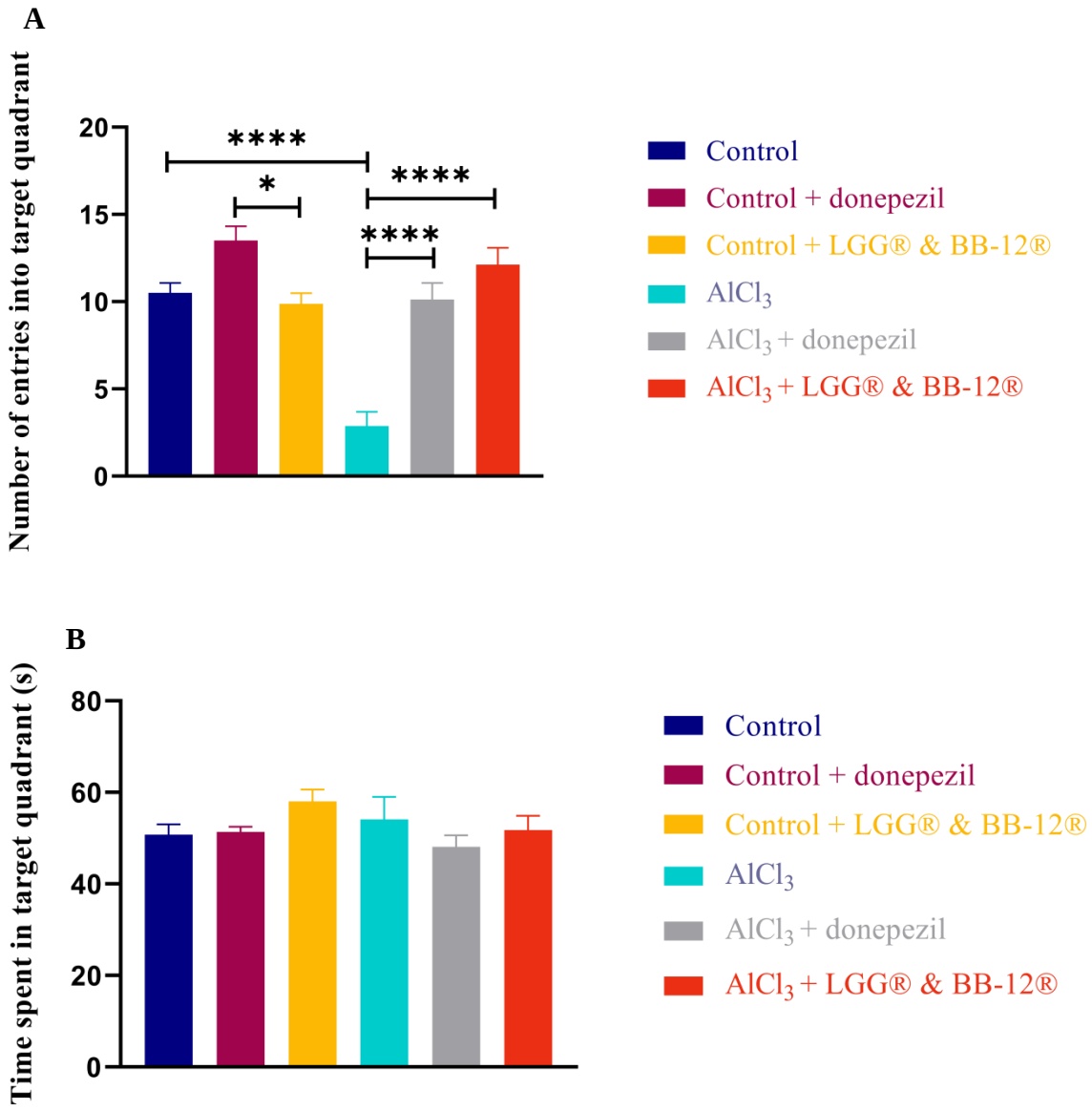

Figure 4.6 The effect of donepezil and LGG®& BB-12® on (A) Number of entries (B) Time spent. Mean ± SEM was used to represent error bars. Statistical analysis was conducted using Graphpad Prism Version 8.0.1.“n.s=p>0.05”, “*=p<0.05”, “**=p<0.01”, “***=p<0.001” and “****=p<0.0001”

4.2 Histological assessment

Histopathological analysis of all study groups was performed by Congo red staining to assess morphological changes in the hippocampus. The Congo red staining revealed an increase in amyloid plaques in $AlCl_3$ group compared to controls and this increase was offset by treatment with donepezil and LGG®& BB-12®. A marked difference was observed in cell bodies in $AlCl_3$ group (31.50±1.32) as compared to control group (66.75±1.75), this decrease was statistically significant ($p<0.0001$). Number of observed cell bodies was significantly more ($p<0.0001$) in $AlCl_3$ + donepezil-treated group (52.5±1.55) and $AlCl_3$ + LGG®& BB-12®-treated group (60.0±2.67) as compared to $AlCl_3$ group (31.50±1.32). Cell counting was conducted at 10X in the dentate gyrus region of hippocampus.

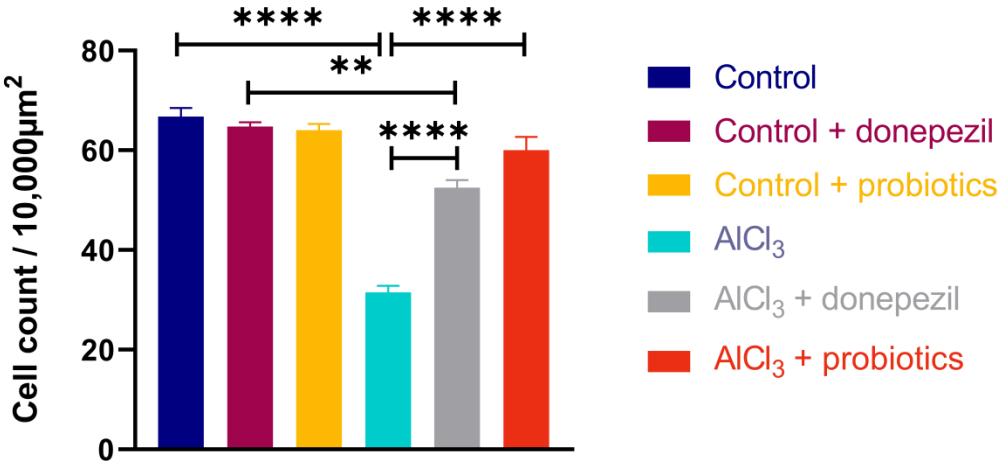

Figure 4.7 The effect of donepezil and LGG®& BB-12®on cell count in dentate gyrus, hippocampus. Mean ± SEM was used to represent error bars. Statistical analysis was conducted using Graphpad Prism Version 8.0.1. "n.s=p>0.05", "*=p<0.05", "**=p<0.01", "***=p<0.001" and "****=p<0.0001"

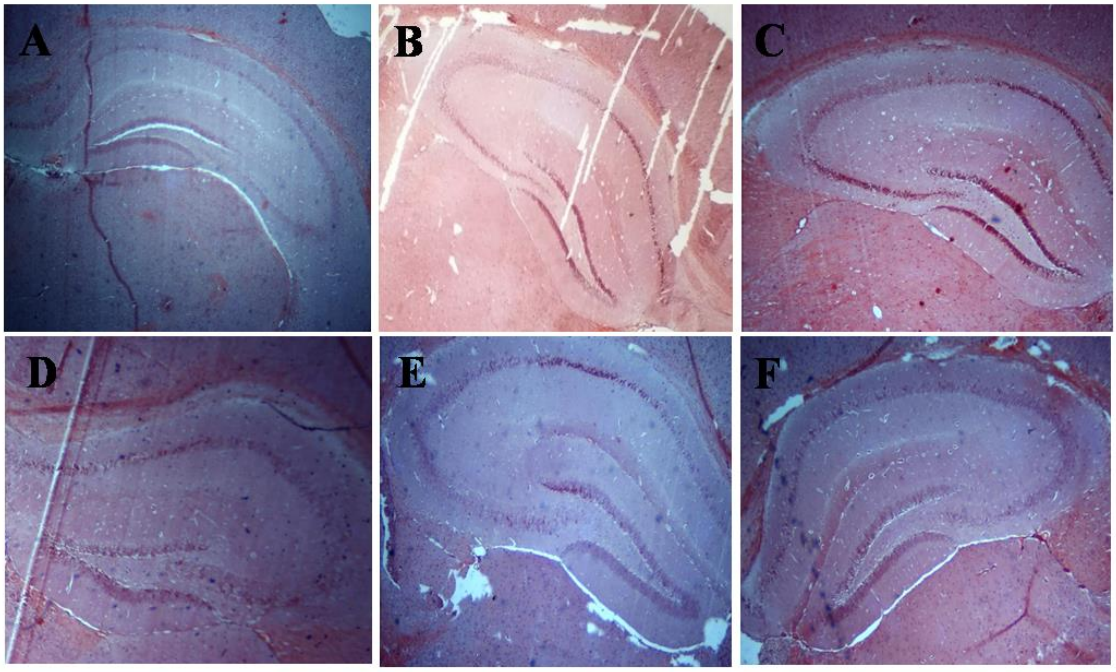

Figure 4.8 Congo red stained coronal sections of hippocampus 4X magnification. (A) Control (B) Control + donepezil (C) Control + LGG®& BB-12® (D) $AlCl_3$ induced AD model (E) $AlCl_3$ + donepezil (F) $AlCl_3$ + LGG®& BB-12®

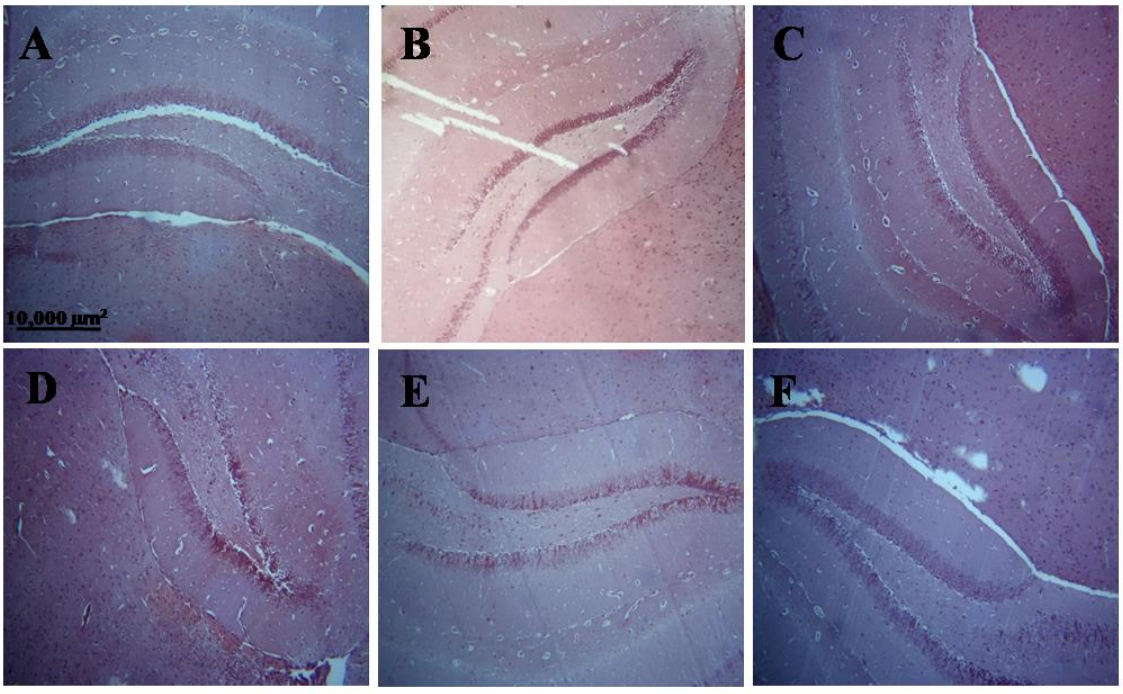

Figure 4.9 Congo red stained coronal sections of hippocampus 10X magnification. (A) Control (B) Control + donepezil (C) Control + LGG®& BB-12® (D) $AlCl_3$ induced AD model (E) $AlCl_3$ + donepezil (F) $AlCl_3$ + LGG®& BB-12®

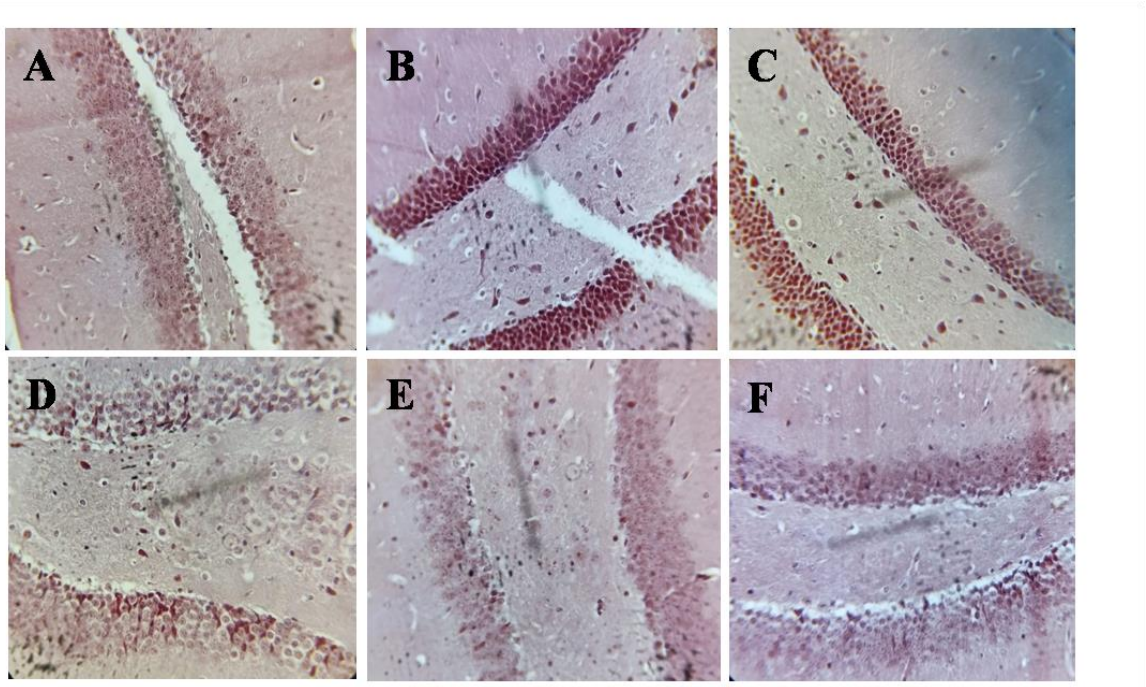

Figure 4.10 Congo red stained coronal sections of hippocampus 40X magnification. (A) Control (B) Control + donepezil (C) Control + LGG®& BB-12® (D) $AlCl_3$ induced AD model (E) $AlCl_3$ + donepezil (F) $AlCl_3$ + LGG®& BB-12®

4.3 Genomic analysis through RT-PCR

4.3.1 Effect of donepezil and LGG®& BB-12®on TNF-α expression

Effect of treatment on expression of TNF-α was determined by performing RT-PCR. Expression of TNF-α was significantly higher ($p<0.0001$) in $AlCl_3$ group (1.98±0.12) as compared to control group (1.00±0.0). Expression of TNF-α in donepezil-treated group (0.65±0.03) and LGG®& BB-12®-treated group(0.65±0.03) was comparatively lower ($p<0.01$) than that of control (1.00±0.0). Similarly, expression of TNF-α in $AlCl_3$ + donepezil-treated group (0.43±0.04) and $AlCl_3$ +LGG®& BB-12®-treated group (0.31±0.01) was significantly less ($p<0.0001$) as compared to$AlCl_3$ group (1.98±0.12). The effect of treatment with donepezil and LGG®& BB-12®on both $AlCl_3$ group and control group was comparable.

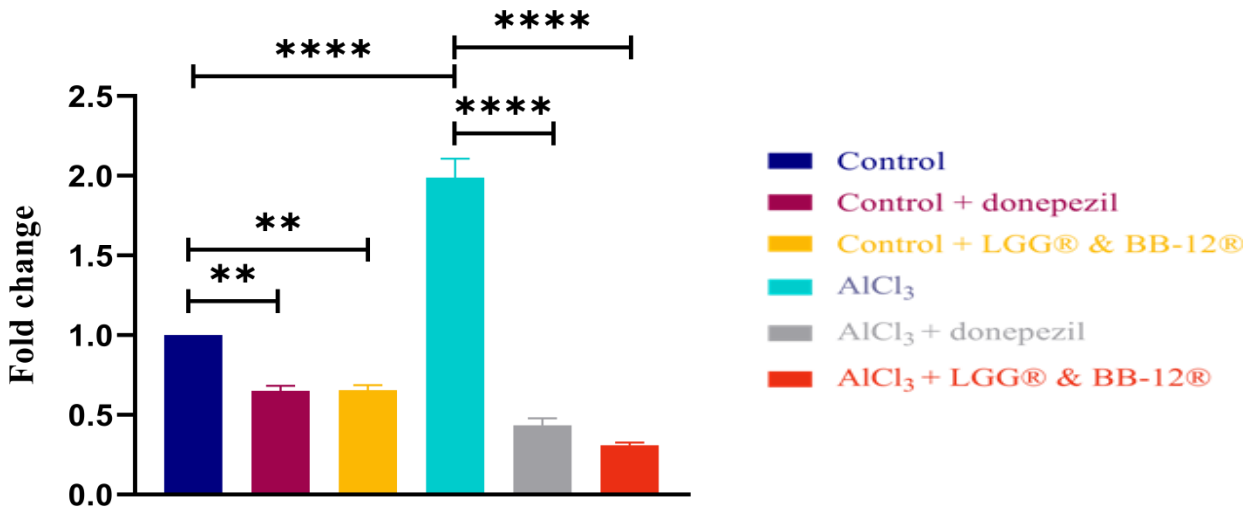

Figure 4.11 Effect of donepezil and LGG®& BB-12® on TNF-α expression. Mean ± SEM was used to represent error bars. Statistical analysis was conducted using Graphpad Prism Version 8.0.1 "n.s=p>0.05", "*=p<0.05", "**=p<0.01", "***=p<0.001" and "****=p<0.0001"

4.3.2 Effect of donepezil and LGG®& BB-12®on IL-1β expression

Expression of IL-1β was significantly higher (p<0.0001) in $AlCl_3$ group (1.22±0.02) as compared to control group (1.00±0.0). Expression of IL-1β was significantly lower(p<0.0001) in donepezil-treated group (0.39±0.02) and LGG®& BB-12®-treated group (0.53±0.01) than control group expression (1.00±0.0). Similarly, expression of IL-1β was significantly less (p<0.0001) in $AlCl_3$ + donepezil-treated group (0.46±0.01) and $AlCl_3$ +LGG®& BB-12®-treated group (0.55±0.01) than in $AlCl_3$ group (1.22±0.02). The effect of treatment with donepezil and LGG®& BB-12®on both $AlCl_3$ group and control group was comparable.

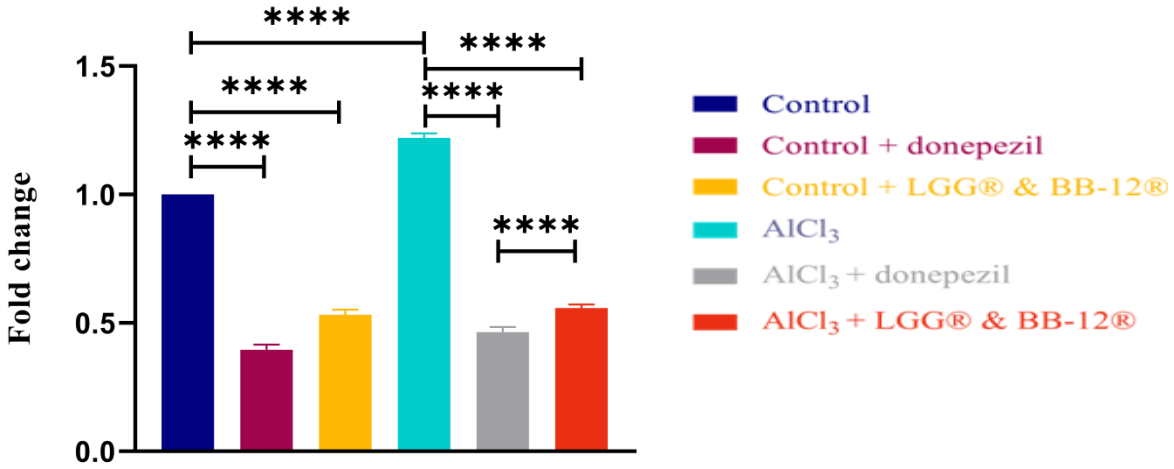

Figure 4.12 Effect of donepezil and LGG®& BB-12® on IL-1β expression. Mean ± SEM was used to represent error bars. Statistical analysis was conducted using Graphpad Prism Version 8.0.1."n.s=p>0.05", "*=p<0.05", "**=p<0.01", "***=p<0.001" and "****=p<0.0001

DISCUSSION

This study investigated the therapeutic effects of combination therapy of probiotics; *Lactobacillus rhamnosus LGG*® and *Bifidobacterium* BB12® on AD mouse models post-$AlCl_3$ treatment (300mg/kg). The treatment was carried out orally for 28 days at a dosage of $1x10^9$ CFU per day. Efficacy of treatment was evaluated by behavioral analysis of anxiety-like behavior and development of memory and learning.

Around 40% patients of AD suffer from anxiety, a common neuropsychiatric symptom in AD (Mendez, 2021). The present study suggested that $AlCl_3$ group exhibited anxiety-like effects as evidenced by number of open arm entries as compared to number of entries made by control group in EPM. In contrast, no significant difference in the time spent in open arms was observed when compared to controls. This suggested that although $AlCl_s$ group displayed anxiety-like behavior and reduced exploratory behavior in EPM the behavior was not starkly different from the behavior of control group.

Donepezil is an ACE inhibitor that produces promnestic effects in humans and rodents but evokes an unclear response when studying its effects on anxiety phenotype (Ben-Azu et al., 2019). Several studies indicate that acetylcholine signaling may contribute in modulation of mood. A study administered physostigmine into hippocampus of C57BL/6J male mice resulting in blockade of ACE activity, an increase in hippocampal acetylcholine which ultimately led to exhibition of anxiety like behavior (Mineur et al., 2013). ACE levels in fluoxetine-treated Swiss-Webster mice were observed to be positively correlated to decrease in anxiety (McCloskey et al., 2017). In another study, donepezil lowered anxiety in transgenic AD mice (Ye et al., 2013). Other studies indicate that donepezil does not affect anxiety but induces hypolocomotion which can be

interpreted as an increase in anxiety (Poole et al., 2014; Soliani et al., 2020). In contrast, an increase in anxiety-like behavior was shown by healthy controls as compared to AD patients being treated with donepezil. This indicates that donepezil administration may display a difference in outcomes post-treatment depending on disease status of individual (Pompeia et al., 2013). Moreover, AD patients exhibiting increased cortisol levels did not show improvement in anxiety post-treatment (San Chang et al., 2018). Shin et al., 2019 establishedthat a dose-dependent response exists in rodents (Sin et al., 2019). A novel study on donepezil in zebrafish exhibited an increase in cortisol levels and anxiety (Giacomini et al., 2020). Ben-Azu et al., 2019 conducted a study in rodents that corroborated the link between stress response and the cholinergic system (Ben-Azy et al., 2019).

Similarly, within the scope of this study a significant increase in anxiety-levels in healthy mice treated with donepezil. No notable improvement was displayed by $AlCl_3$ + donepezil-treated group.

Probiotics oral administration improves anxiety-like behavior in both animal and human studies. C57BL/6 pregnant female mice were orally gavaged with *Lactobacillus rhamnosus* LGG®from the 18th day of gestation until birth and offspring were also gavaged for 5 days. LGG® colonization resulted in increased expression of GABA receptors, BDNF and serotonin transporter leading to anti-anxiety like behavior in adulthood (Zhou et al., 2022). Male mice of C57BL/6 strain were administered *Lactobacillus rhamnosus* (JB-1) for a period of 28 days followed by chronic social defeat. Mice treated with probiotics had a decreased anxiety-like response to social stress as compared to placebo group. JB-1 colonization resulted in increased IL-10 regulatory

T-cells and reduced activation of dendritic cells (Bharwani et al., 2017). Sprague-Dawley rats treated with *Lactobacillus rhamnosus* LGG® and pre-biotic combination after experiencing maternal separation showed improvement in anxiety-like behavior with a decrease in mRNA expression of GABA A2 in comparison to untreated rats (Neufeld et al., 2017). 423 women in Auckland participated in a clinical trial in which they were administered probiotic *Lactobacillus rhamnosus* (HN001) from time of recruitment till 6 months post-partum. Women receiving probiotic treatment exhibited lower anxiety scores as compared to placebo group. It was concluded that the probiotic regimen may be a potential therapeutic route to attenuating anxiety and depression in mothers postpartum (Slykerman et al., 2017). A single arm study, investigated the effect of consumption of *Bifidobacterium* BB12®on state anxiety in athletes. The intervention resulted in significant improvement in somatic anxiety, emotion anxiety and cognitive anxiety (Dong et al., 2021). This study corroborated the existing evidence that probiotics are helpful in attenuating anxiety-like behavior. Combination therapy of *Lactobacillus rhamnosus* LGG® and *Bifibobacterium*BB12®resulted in significant decrease in anxiety like behavior in both control + LGG®& BB12®treated group and $AlCl_3$ + LGG®& BB12®group. Reduced anxiolytic behavior was indicated by an increase entries and time spent in open arm. LGG®& BB12®treatment is effective in decreasing anxiety-like behavior associated with AD.

Several parameters of memory including spatial memory, reference memory and long-term spatial memory were studied by employing different behavioral tests to better determine the effect of probiotic treatment on deficits of memory and learning in AD as different procedures target distinct idiosyncrasies within a domain. Donepezil has

promnestic effects and donepezil administration at 3mg/kg and 10mg/kg prevented memory impairment induced by scopolamine in hairless rats as evidenced by their performance in Y-maze. Rats pre-treated with donepezil before scopolamine intraperitoneal injection displayed significantly higher % spontaneous alternation than those that received no pre-treatment (Shin et al., 2018). APP/PS-1 transgenic mice had significantly improved performance in NOR and MWM following administration of donepezil by attenuating expression of CD68 which is involved in microglial activation and IL-1β and TNF-α(Guo et al., 2015). The current study indicated that $AlCl_3$ + donepezil-treated group had improved performance in % spontaneous alternation, DI and escape latency. Therefore, it was effective in ameliorating cognitive deficits induced by $AlCl_3$.

Lactobacillus rhamnosus UBLR-58 potentiated activity of curcumin in ameliorating dementia by increasing the bioavailability of curcumin (Patel et al., 2020). Combination therapy using *Lactobacillus rhamnosus, Bifidobacterium infantis* and *Lactobacillus reuteri*for treatment of AD induced by Aβ1-40 intra-hippocampal injection led improved spatial memory. This improvement was assessed by MWM. The improvement was rendered by a decrease in oxidative stress and inflammation in probiotic treated group (Mehrabadi& Sadr, 2020). Administration of *Bifidobacterium Lactis* Probio-M8 to APP/PS-1 transgenic mice led to an increase in α and β- diversity of gut. Treated mice also displayed significant improvement in % spontaneous alternations and % number of contacts with the novel object, revealing that treatment with probiotics improved cognitive decline in APP/PS-1 transgenic mice (Cao et al., 2021). AD was induced in male Wistar rats by injecting Aβ1-42 into intra-cerebro-ventricular region of brain.

Treatment using probiotic combination of *B. bifidum* and *L. plantarum* with exercise led to attenuation of cognitive decline particularly in spatial learning and memory (Shamsipour et al., 2021). Middle aged to older adults exhibiting symptoms of cognitive decline were enrolled in a clinical trial and supplemented with *Lactobacillus rhamnosus* leading to an improvement in the cognitive decline.

In the present study, $AlCl_3$-treated group displayed a significant decrease in cognition and had poor performance on all three memory tests. A significant decrease was observed in both spatial and reference memory parameters. Long-term spatial memory and learning was also poor as observed in escape latency. During the MWM $AlCl_3$-treated group showed an atypical behavior i.e., the time spent in target quadrant was not less as compared to other groups although clear memory impairment was observed. This was due to the presence of "floaters". Floaters are animals that due to their anxious behavior display hypolocomotion. Therefore, MWM results must be elaborated carefully with all four parameters within MWM being considered concomitantly. $AlCl_3$ + LGG®& BB12®displayed a significant improvement in all parameters of memory tests. The improvement in cognition was comparable to that of control. It can be concluded that this combination of probiotic treatment is effective improving cognitive decline in AD.

Coronal sections of hippocampus were stained using Congo red. Congo red dyes amyloid-β plaques a dark red color and stains the nucleus of neurons a blue-purple color. Observation of stained sections under a light microscope showed an increase in the number of amyloid-β plaques in $AlCl_3$ group as compared to all other groups. $AlCl_3$ + donepezil-treated group and $AlCl_3$ + LGG®& BB12® displayed less plaque deposition. Cell counting conducted under 10x magnification showed a marked decrease in cell

bodies in dentate gyrus in $AlCl_3$ group. $AlCl_3$ increases oxidative stress, inflammation and reactive gliosis leading to neurodegeneration (Prakash et al., 2013). Treatment with donepezil and probiotics resulted in cell counts comparable to that of control group. Donepezil induces hippocampal neurogenesis in AD and following traumatic brain injuries (Yu et al., 2015). Donepezil induces neurogenesis by modulating BDNF/TrkB dependent signaling and regulating the cholinergic system. It increases expression of BDNF and levels of phosphorylated TrkB (Zheng et al., 2018). Zebrafish treated with *Lactobacillus rhamnosus* had an increased expression of BDNF (Cuomo et al., 2021). LGG® affected BDNF expression in Wistar rats with enteropathy and the BDNF expression was higher and TrkB levels were lower (Orlando et al., 2021). Treatment with a combination of *B. bifidum* and *L.plantarum* increased expression of BDNF and ChAT in AD rats (Shamsipour et al., 2021).

An increase in expression of TNF-α and IL-1β was seen in AlCl3 group. Increase in systemic inflammation mediated by pro-inflammatory cytokines plays major pathophysiological role in AD development (Gonzalez-Reyes et al., 2017). There was a significant decrease in cytokine expression in both $AlCl_3$ + donepezil and $AlCl_3$ + LGG®& BB-12® group. Several studies show similar effects post-probiotic treatment. AD rats treated with *Lactobacillus rhamnosus, Bifidobacterium infantis* and *Lactobacillus reuteri* showed significant decrease in TNF-α and IL-1β as shown by ELISA tests (Mehrabadi& Sadr, 2020). Treatment of adult zebrafish with Lactobacillus rhamnosus modulated expression of cytokines (Bootorabi et al., 2021). Bifidobacterium BB12® attenuated TLR2 cytokine expression in young healthy adults (Meng et al., 2017).

CONCLUSION AND FUTURE PROSPECTS

The present study proved that probiotic treatment was effective in improving cognitive decline and anxiety-like behavior associated with AD. Since, probiotics are relatively cheap and well-tolerated; they can serve as a potential treatment option in the future. The gut microbiota-brain axis is complex and must be further explored to exactly determine how these probiotics were so effective in treatment of AD. Future prospects include 16s RNA profiling of fecal microbial diversity to determine the effect of probiotic oral consumption on α-diversity of microbes in gut. Furthermore, effect of probiotic consumption must also be studied on oxidative stress and neurogenesis to give further insight as to how gut microbiota attenuates the severity of disease symptoms.

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