The therapeutic effect of *Limosilactobacillus fermentum* strains and chrysin in chronic unpredictable mild stress mice model

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DEDICATION

All My Effort is dedicated to the
"MY BELOVED FAMILY"
Especially my Father and Mother

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I would close this chapter with a verse from the Holy Quran *"And my success (in my task) can only come from Allah. In Him I trust, and unto Him I look"* (11:88 Al-Quran).

Iqra Mutiullah

Table of Contents

List of Figures

List of Tables

List of abbreviations

ACTH: Adrenocorticotrophic hormone

ADRs: Adverse drug reactions

BDNF: Brain-derived neurotrophic factor

BP: Bipolar disorder

CNS: Central nervous system

CRF: Corticosterone-releasing factor

DC: Diseased Control

DA: Dopamine

ENS: Enteric nervous system

EPM: Elevated maze plus

FST: Forced swim test

GABA: γ-Aminobutyric acid

GBA: Gut brain axis

GR: Glucocorticoid receptor

HPA: Hypothalamic-pituitary-adrenal axis

H&E: Hematoxylin and eosin

LAB: Lactic acid bacteria

MWM: Morris water maze

MMD: Major depressive disorder

MAO: Monoamine oxidase

MAOI: Monoamine oxidase inhibitor

MIS: Mucosal immune system

NE: Norepinephrine

NET: Nucleic acid testing

OBX: Obesity, Susceptibility to, X-Linked

MR: Mineralocorticoid receptor

NC: Negative control

PC: Positive control

PFC: Prefrontal cortex

OPT: Open field test

SPT: Sucrose preference test

SSRIs: Selective serotonin reuptake inhibitors

SNRIs: Serotonin, Noradrenalin Reuptake Inhibitors

SERT: Serotonin reuptake transporter

TCA: Tricyclic antidepressants

TP: Probiotic treatment group

TC: Chrysin treatment group

TPC: Probiotic and chrysin treatment group

VN: Vagus nerve

YLD: Years to lost disability

WHO: World health organization

Abstract

Depression is a chronic neuropsychiatric disorder and it has an impact on the lives millions of people globally. It can strike at any time in life, from infancy to old age, causing considerable anguish and disruption of life, and ultimately leading to death if ignored. Currently, there is no cure for depression, but there are a variety of treatment options that can help keep symptoms at bay. The human gut microbiota influence the production of neuromodulators such as serotonin, dopamine, GABA, BDNF and SCFAs thus helps in regulating the gut-brain axis. This study investigated the effect of psychobiotics by oral consumption of combination of *Limosilactobacillus fermentum* strains along with Chrysin on CUMS induced depression mouse models in comparison with fluoxetine. The mice were randomly divided into six groups. The Sucrose Preference Test (SPT), Elevated Plus Maze (EPM), Open Field Test (OFT), Forced Swim Test (FST) and Morris Water Maze (MWM) were used to evaluate the impact of probiotics and chrysin therapy on anxiety and depression. The results showed that psychobiotics i.e., treatment with probiotics and chrysin (TPC) was shown to significantly ($p<0.0001$) lessen the effects of CUMS on anxiety and depression. Histopathological assessment performed through H&E staining showed remarkable decrease in cell necrosis and cell shrinkage burden within the mice hippocampus observed in the group treated with TPC (combination of probiotics consortium and chrysin) subjected to CUMS. Remarkable difference was also observed in H&E staining of colon. There was no epithelial crypt loss, villus atrophy and cell infiltration observed in TPC group as compared to disease group. Thus, the present findings indicate that psychobiotics i.e., probiotics and chrysin have strong potential to be used as combination therapy for depression by modulating the gut microbiota-brain axis.

Keywords: Probiotics, chronic unpredictable mild stress, depression, microbiota, gut-brain axis, psychobiotics, *Limosilactobacillus fermentum*, inflammation, polyphenols, chrysin

CHAPTER 1

1.0 Introduction

Depression is an incredibly widespread and debilitating mental health issue that affects approximately 280 million individuals worldwide (Miller & Raison, 2016). It has been identified as a major global cause of disability, with a 3.8% prevalence rate among the population-particularly 5% in adults aged 15 to 29 (Kessler & Bromet, 2013). Not only can depression have severe consequences if not addressed, such as suicide - which annually claims 700,000 lives globally – it also manifests differently for each individual due to the many hidden causes: genetic factors; neurological disturbances; inflammation issues; psychological stressors; cognitive deficits or environmental influences (Metrics & Evaluation 2021) (Wohleb et al., 2016a).

Various treatment approaches exist for people experiencing depression, such as prescription medications and psychotherapy. Medications work to adjust the neurological functioning underlying depressive symptoms, while psychological therapies focus on identifying unhelpful thought patterns associated with depression and replacing them with healthier strategies (Wallace & Milev, 2017). Research shows that between 60-70% of patients who undergo these treatments observe a reduction in depressive symptoms as a result (Al-Harbi, 2012). However, current treatments only work for around two-thirds of patients, and many people avoid getting help because of the stigma associated with the disease (Rieder et al., 2017). Therefore, additional or alternative treatment methods are required for depression. Thus, depression requires supplementary or complementary therapy options.

The gut microbiota significantly influences human health and illness (Gareau et al., 2010) (Madison & Kiecolt-Glaser, 2019). Beyond the gastrointestinal tract, it has been connected to conditions including obesity (Turnbaugh et al., 2009), celiac disease (De Palma et al., 2010), Type-2 diabetes (Larsen et al., 2010), Crohn's disease (Scanlan et al., 2006), and depression (Lin et al., 2017). Numerous studies in

both rodents and people show that changes to the gut microbiota increase stress reactivity and are linked to negative mental health consequences. The evidences connecting the gut microbiota and mental health is becoming stronger with faecal microbiota transplants (FMTs). In experiments on mice, BALB/c animals displaying anxious behaviours have been seen to switch towards a more active and confident behaviour after receiving FMT from NIH Swiss donors. Likewise, FMTs from sad individuals cause mice to exhibit depressive behaviour (Zheng et al., 2016) (J. R. Kelly et al., 2016). These results suggest that the gut microbiota has a major effect on mental health and that microbiome manipulation could be a therapeutically viable alternative.

Dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis, which controls the body's reaction to stress, has been related to depression (Foster et al., 2017). Research suggests that this dysregulation can lead to chronic inflammation and alterations in gut microbiota composition, which can further exacerbate depressive symptoms (Slyepchenko et al., 2017). The gut microbiota has been shown to influence the HPA axis by controlling inflammation and the release of neurotransmitters and hormones (Kelly et al., 2016). Thus, altering the composition of the microbiota in the digestive tract may be an effective strategy for combating depression.

The interaction between the gut and the brain to influence both physical and mental health is called ‘gut-brain-axis’. This communication pathway has two distinct yet closely linked components- gastrointestinal tract (GI) & central nervous system (CNS). It synchronizes hormonal, neuronal and immunological signals from both these organs in order to regulate mental wellbeing as well as promote a healthy lifestyle. The microbiome of our digestive systems also plays an essential role with respect to how this bi-directional connection can be transduced into effecting change in one's overall life satisfaction levels (Mayer, Knight, et al., 2014) (Steenbergen et al., 2015). Studies conducted on mice have demonstrated that probiotics can produce antidepressant effects, but these are lost when the vagus nerve is cut off. This indicates there must be communication between the stomach and brain through this particular nerve in order for these beneficial effects to occur (Bravo et al., 2011a). Tryptophan

metabolites are an additional potential pathway implicated in the gut-brain relationship. The majority of tryptophan is converted to kynurenine instead of serotonin, particularly under inflammatory circumstances. Kynurenine may be broken down into the neuromodulatory fatty acids anthranilic acid, kynurenic acid, and quinolinic acid. (Kennedy et al., 2017a) (Waclawiková & El Aidy, 2018). Research indicates that there is a link between our gut microbiome and mental health. It appears that certain types of bacteria in the digestive tract can play a role in regulating inflammation, which may have an effect on mood disorders such as depression. Studies have linked the systemic inflammation caused by these bacterial populations and poor mental health outcomes, with elevated levels of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-alpha) among those suffering from depression. One possible explanation for the increased inflammation is a "leaky gut" (Maes et al., 2012), albeit this remains unproven. A decrease in barrier function, which includes a mucus layer and complex tight junctions on the epithelial cells, leads to an increase in gut permeability, which is characteristic of a leaky gut. Different bacterial species have been shown to have opposing effects on this barrier, strengthening it for some while weakening it for others (Pedicord et al., 2016). The intestinal barrier is critical for the maintenance of health, as it prevents various bacteria and their toxic products from entering the central circulation (Maes et al., 2012). When this natural wall is compromised, known as gut permeability, compounds such as lipopolysaccharide (LPS) can enter into systemic circulation and cause low-grade inflammation in multiple organ systems including brain function. This pro-inflammatory response has been linked to disrupted serotonin transmission resulting in depressive symptoms (Sampson & Mazmanian, 2015).

In recent years, the notion of "psychobiotics," defined as probiotics that can promote mental health, has arisen (Sarkar et al., 2016). Probiotics are described as bacteria that are alive and give a health benefit (Hill et al., 2014a). Animal and human preclinical research suggest that probiotic medication improves behavior and mood, and there is evidence that probiotic bacterial species promote gut barrier function (Steenbergen et al., 2015). Psychobiotics are a type of probiotic supplement that includes live bacterial

strains, like *Limosilactobacillus* and *Bifidobacterium* species. These bacteria interact with the gut-brain axis - which is the two-way connection between your mental wellbeing and body - to potentially influence behaviour or brain function beneficially. Evidence suggests that modifying this system may actually assist in managing symptoms associated with various mental health issues such as depression.

A recent study published in the journal Nutrients has suggested that psychobiotics could be useful for treating symptoms of depression, anxiety and stress. The research involved giving a multi-strain probiotic supplement containing *Limosilactobacillus* and *Bifidobacterium* species to adults who had major depressive disorder (MDD). Results revealed improvement in mood and anxiety during the duration of the experiment. This suggests that taking probiotics may help reduce feelings associated with MDD, providing further evidence as to their potential benefits when it comes to mental health (Wallace & Milev, 2017). Another study published in "Brain, Behavior, and Immunity" found that a probiotic supplement containing *Bifidobacterium longum* reduced symptoms of depression and improved cognitive function in healthy volunteers (Takagi et al., 2019). The effects psychobiotics have on the brain remain unclear, though it is believed their action involves different pathways. These may include increased release of neurotransmitters like serotonin and GABA, regulation of hormones through modification to the HPA-axis, and modulation of inflammation and oxidative stress (Hill et al., 2014a).

The use of probiotics to promote health and wellbeing has a long history, as documented by the ancient Greeks and Romans who recommended consuming cheese and fermented foods (Gismondo et al., 1999). With further research being conducted on these beneficial strains, it is now thought that they can reduce gastrointestinal discomfort such as flatulence or bloating while also helping with bowel regularity. It has been widely reported that probiotics could protect against damage from oxidation due to DNA, proteins or lipids whilst bolstering both skin function & immune systems which leads to fewer allergies over time in addition lessening infection levels (Jafarnejad et al., 2016) (Zhang et al., 2016). The

importance of healthy microbial content within our intestines - otherwise knownas 'gut microbiota'- impacting human emotions have recently become apparent through investigation into bidirectional communication between gut bacteria/microbes & brain activity (Dinan&Cryan; 2013); there are increasing reports claiming people's feelings may be influenced by bacterial species residing in their guts (Schmidt 2015)). This interesting notion links psychology & gastrointestinal composition meaning researchers must continue unravelling more about how certain microorganisms affect behaviour as well neuro development alongside influencing immunity too (Ogbonnayaetal2018) (Mu et al., 2016) .

Studies suggest that there may be a relationship between the composition and function of gut microflora with depression. This could potentially come from communication pathways such as the gut-brain axis, which allows bi-directional conversations to take place between our central nervous system and enteric nervous system. Consequently, research has implicated *Limosilactobacillus fermentum* - a species of lactic acid bacteria - in providing beneficial effects on not only gut health but also immune functioning, when studying these possible links with mental health disorders like depression. The potential for *L. fermentum* to improve mental health has been highlighted by recent research, with benefits including reduced symptoms of anxiety and depression. The effects of a probiotic supplement containing *Limosilactobacillus fermentum* on the gut microbiota and depressive symptoms in people with major depressive disorder were investigated in a research set to be published in 2020. The study found that the probiotic supplement led to significant changes in the gut microbiome and improvements in depressive symptoms, possibly mediated by the HPA axis (Tremblay et al., 2021). Another study published in 2020 investigated the effects of *Limosilactobacillus fermentum* on stress-induced behavioral and neurochemical changes in rats. *Limosilactobacillus fermentum* was discovered to have a therapeutic effect in stress-related mental diseases, according to a recent study (Farzi et al., 2018). This was due to the fact that it was able to minimize stress-induced alterations in the HPA axis and enhance behavioral outcomes. A study published in 2019 investigated the effects of *Limosilactobacillus fermentum* on depressive-like behavior and inflammatory response in rodents exposed to chronic, unpredictable, mild

stress. The study found that *Limosilactobacillus fermentum* administration led to reduced depressive-like behavior and decreased inflammatory response, potentially mediated by the gut-brain axis (Wang et al., 2015).

Animal studies indicate that probiotics may boost plasma tryptophan levels and decrease serotonin and dopamine concentrations in the frontal brain and cortical dopamine metabolites, hence alleviating depression symptoms (Akkasheh et al., 2016) (Desbonnet et al., 2008). The effects of probiotics on human health, particularly psychiatric illnesses, have lately attracted the attention of neuroscientists. Moreover, new research suggests that probiotics may have an impact on mood. Although a bad diet has been demonstrated to be a risk factor for depression, one would anticipate a good diet to have a preventative impact on depression. Dietary regulation of probiotics could have important implications for the prevention and treatment of depression (Evrensel & Ceylan, 2015).

Another alternative remedy to alleviate depression is the use of dietary polyphenols. Polyphenols are a class of bioactive compounds found in plants that have antioxidant and anti-inflammatory properties. They are abundant in fruits, vegetables, tea, coffee, cocoa, and wine, among other sources (Manach et al., 2004). Polyphenols are divided into several subclasses, including flavonoids, phenolic acids, stilbenes, lignans, and others. Studies indicate that a diet high in polyphenols may decrease the risk of developing chronic conditions, like cardiovascular disease, cancer and neurodegenerative disorders (Scalbert et al., 2005).

Research is showing that polyphenols may help promote better mental health through reducing depression and anxiety symptoms. It has been discovered that these compounds can influence the levels of certain neurotransmitters, like serotonin, dopamine, and norepinephrine; all three are essential for regulating moods (Tayab et al., 2022a). It has been demonstrated that flavonoids such as chrysin, quercetin, and kaempferol increase serotonin levels in the brain by inhibiting monoamine oxidase, an

enzyme that degrades serotonin. This mechanism is comparable to that of commonly prescribed antidepressants known as selective serotonin reuptake inhibitors (SSRIs) (Tayab et al., 2022a).

Chrysin is a natural flavonoid that can be found in several plant sources such as passionflower, honey, and propolis. Recent studies have suggested that chrysin can reduce symptoms of depression by increasing serotonin levels in the brain. Serotonin is a neurotransmitter that regulates mood, anxiety, and stress response (Bortolotto et al., 2018). By inhibiting the activity of the serotonin transporter, chrysin blocks the reuptake of serotonin, leading to an increase in its availability and a reduction in depressive symptoms. Additionally, chrysin has been shown to have anxiolytic and sedative effects by modulating the activity of the GABA-A receptor, a neurotransmitter receptor involved in anxiety and sleep regulation (Nabavi et al., 2015a).

Foods containing polyphenols, such as fruits and vegetables, have been linked to lower rates of mental health disorders such as melancholy and anxiety. Research suggests that these compounds can modulate the activity of neurotransmitters involved with mood regulation by affecting their synthesis or degradation levels within the brain. Furthermore, polyphenols may also affect other components related to emotional wellbeing - such as gut microbiota composition and hypothalamic-pituitary adrenal (HPA) axis function – both of which are known for playing a crucial role in regulating our cognitive responses and behavior including feelings associated with depression or anxiety (Tayab et al., 2022b). Polyphenols have been found to shift the balance of gut microorganisms in a positive direction. This can involve stimulating growth in probiotic bacteria such as *Limosilactobacillus* and *Bifidobacterium*, while also suppressing populations of pathogenic bacteria including Clostridium and Escherichia coli. This modification of the intestinal microbiota can result in the production of metabolites, such as short-chain fatty acids and tryptophan, which have antidepressant and anxiolytic effects (Rodrguez-Landa et al., 2022).

The Hypothalamic-Pituitary-Adrenal (HPA) Axis is a complex system that regulates the body's stress response. Prolonged tension can interfere with its functioning, leading to elevated cortisol levels, increased inflammation and oxidative pressure; all symptoms associated with depression and anxiety (Pathak et al., 2013). Polyphenols have been known to reduce these effects by suppressing cortisol production as well as lessening inflammation whilst boosting antioxidant enzymes such as Superoxide Dismutase and Catalase (Winiarska-Mieczan et al., 2023).

By modulating neurotransmitters, the gastrointestinal microbiome, and the HPA axis, polyphenols may have a beneficial effect on mental health, specifically in reducing the symptoms of depression and anxiety.

1.1 Aims and Objectives

The purpose of the present study was to evaluate the therapeutic effects of oral administration of combination of *Limosilactobacillus fermentum* consortium (7b, 19, and 18) and chrysin on the CUMS mouse model of depression as compared to standard drug fluoxetine.

Objectives:

- Analyze the effects of *L. fermentum* strains and chrysin on anxiety and depression displayed by CUMS mice model via behavioral tests.
- Compare the effect of worldwide commercially available drug (fluoxetine) with probiotics and chrysin.

CHAPTER 2

2.0 Literature Review

Depression is a highly serious condition that has the potential to be fatal, impacting a significant number of individuals globally. It can manifest at any stage of life, spanning from early childhood to late adulthood, and places a substantial burden on society. This mental illness inflicts immense suffering and upheaval in individuals' lives, and if left untreated, it can have fatal consequences (Brigitta, 2002). Depression is a chronic neuropsychiatric condition characterized by a high likelihood of reoccurrence, impacting over 350 million people worldwide. This disorder carries significant burdens on both public health and the economy (Ledford, 2014). As the fourth main cause of disability, depression carries the greatest proportion of the overall burden of illness. It accounts for 10.3% of the total disease burden and results in an astounding 76.4 million years lost to disability (YLD) globally (Friedrich, 2017). Depression is a complex phenomenon characterized by various subtypes and likely influenced by multiple factors. It is marked by episodic and often progressive mood disturbances, exhibiting a wide range of symptoms that can range from moderate to severe, occasionally involving psychotic features. Additionally, depression is associated with connections to various psychiatric and physical illnesses (Duman, 2014). The Diagnostic and Statistical Manual in 2000 demarcated the symptoms of depression, based on which depression is diagnosed as “major depression”. Major symptoms given by the Diagnostic and Statistical Manual in 2000 are shown in following (Schatzberg & Nemeroff, 2017).

Symptoms of depression

- Depressed mood (Sadness)
- Irritation (Anger)
- Low-self regard
- The Feeling of melancholy, worthlessness, and delinquency

- Weight change (Gain or loss)
- Insomnia or hypersomnia
- Low energy, exhaustion, or increased anxiety
- Appetite changes (Increase or decrease)
- Loss of interest in enjoyable experiences
- Frequent contemplation of death and suicidal ideation

When a few of the above symptoms affect normal social and occupational performance and are reported for more than two weeks, then the disorder is diagnosed as major depression (Vahia, 2013).

2.1 Prevalence of depression:

Depression has a global lifetime prevalence that can reach up to 20%, with women being affected approximately five times more often than men. Based on a 2019 report by the World Health Organization (WHO), over 20 million individuals in Pakistan, accounting for 10% of the country's population, experience depression (MDD) (Nisar et al., 2019). It is estimated that only about one-third of patients with depression receive therapy, not necessarily due to lack of awareness, but possibly because the symptoms may not appear significantly different from ordinary life experiences. Depression often follows a recurrent pattern, but the majority of individuals eventually recover from major depressive episodes (Douglas et al., 2018). Nevertheless, a significant portion of patients experience chronic depression, with research indicating that 12% of individuals remain depressed after a 5-year follow-up, and 7% still exhibit symptoms after a 10-year follow-up (Kessing & Bukh, 2017). Patients who have successfully recovered from depression face a substantial risk of recurrence, with approximately 75% of individuals experiencing more than one episode of severe depression within a 10-year period (Farb et al., 2015). Depression carries a significant risk of suicide, particularly among young individuals aged 15 to 24, where the suicide rate is notably elevated (Wong & Licinio, 2001). Multiple lines of evidence strongly support the existence of a significant relationship between depression

and cardiovascular disease, as well as increased mortality rates. Numerous studies have shown that depression increases the risk of developing coronary artery disease and impairs the prognosis after a heart attack (myocardial infarction) (Bradley & Rumsfeld, 2015). Moreover, depression has been found to increase the risk of cardiac-related mortality regardless of the initial cardiac health status. Notably, individuals with severe depression face a mortality risk that is more than twice as high as those with moderate depression (Gathright et al., 2017).

The high prevalence of comorbidity with other mental disorders is an additional key component of depression. Anxiety, particularly panic disorder, is frequently related with affective disorders, although the relationship with alcoholism or drug dependence is less. Intriguingly, anxiety typically precedes the start of depression, but alcohol consumption is equally likely to precede or follow the beginning of depression (Cohen et al., 2015) (Merikangas et al., 1996).

2.2 Classification of depression

Depression's etiology and categorization have been the subject of extensive study throughout history. At the start of the 20th century, two competing perspectives—Sigmund Freud's understanding of grief as expression of repressed anger and Emil Kraepelin's view of depression as a disease—represented the field. By categorizing clinical depressive symptoms (ranging from mild psychosis to severe psychosis) into discrete groups of "endogenous" and "reactive" subtypes of depression, Sir Martin Roth and the Newcastle Group added to our understanding of depression (Garside et al., 1971). This approach was utilized for decades in biological psychiatric research to identify subgroups of the condition with different etiologies. Broadly speaking depression is classified into the following main categories:

2.2.1 Major depressive disorder (MDD)

MDD is a mood disorder marked by severe and persistent emotional symptoms, including the sensation of delinquency and distress; poor self-respect; and somatic symptoms, including loss of vigour, sleeplessness, mental capabilities, and exhaustion (Otte et al., 2016).

2.2.2 Minor depressive disorder:

Minor depressive illness is defined by at least two weeks of symptoms leading to functional impairment and suffering. The distress is not caused by a physical condition, substance misuse, or grief (Alexopoulos, 2005).

2.2.3 Bipolar disorder (BD):

BD is characterized by the occurrence of alternating manic and depressed episodes. Manifestations of a manic episode include grandiosity, impatience, overconfidence, talkativeness, a diminished need for sleep, and an extremely elevated mood. Psychotic symptoms, such delusions and hallucinations, are also present during a manic episode. While the depressive episode is marked by depressed mood, exhaustion, and abnormalities in sleep patterns (increased or decreased), low self-esteem and depression (Carvalho et al., 2020) (Grunze, 2015).

2.2.4 Psychotic depression:

It is characterized by severe depression accompanied by psychotic symptoms such as delusions and/or hallucinations, obsession, and false beliefs (Wijkstra et al., 2013).

2.2.5 Melancholia:

It is a severe form of depression marked by psychomotor slowness and extreme mood disruption. Significant physiological disturbances include day-to-day mood volatility, early morning awakening,

weight loss, psychomotor impairment, and malformation of the hypothalamic-pituitary-adrenal (HPA) axis (Jansson, 2020).

2.2.6 Postpartum depression:

Postpartum depression is predicted by a history of stressful life events, psychological suffering during pregnancy, mood disorder, limited social support, and a poor marital relationship (Limandri, 2019).

2.2.7 Peripartum depression:

Peripartum depression is characterised by the following symptoms: stomach discomfort, gloomy mood, headache, breast tenderness, and exhaustion. Women with minor symptoms are advised on lifestyle modifications. Women with moderate symptoms should be educated on lifestyle modifications such as a nutritious diet, regular exercise, decreased stress and anxiety, and decreased salt and caffeine use (Dekel et al., 2019).

2.3 Pathophysiology of depression

The most recognized role in mediating depressive-like behavior is of the hippocampus (MacQueen & Frodl, 2011). Various functions of the brain like learning, anxiety, somatic functions, HPA-axis functioning, and cognitive functions are dysregulated in mood disorders and are mediated by Hippocampus. A few symptoms of depression, such as deficiencies in emotion and cognition, are mediated via connections between the hippocampus and the amygdala and prefrontal cortex (PFC). Stress is one of the factors that can trigger mood-related disabilities via enhancement of neuronal atrophy and reduction of neurogenesis in the hippocampus. Antidepressant treatment improves these cognitive, emotional, and somatic symptoms by reversal of cellular changes in various regions of the brain like the hippocampus. Stress-induced depression is thought to be caused by dysregulated HPA-axis activity (X. Du & Pang, 2015). The dysregulated HPA-axis activity, structural alterations in the brain regions,

neuronal atrophy, reduced neurotrophic growth factors, and reduced neurogenesis are the causes of the pathophysiology of depressive behaviors (Wohleb et al., 2016b).

2.3.1 Mechanism of stress-induced depression

Dysregulation of the HPA-axis is an important mechanism by which either acute or chronic stress affects the brain. The periventricular nucleus neurons of the hypothalamus secrete corticotrophin-releasing factor (CRF). The CRF stimulates the production and secretion of Adrenocorticotrophic hormone (ACTH) by acting on the anterior pituitary. By stimulating the adrenal cortex with ACTH, glucocorticoids (Cortisol in humans and Corticosterone in rodents) are produced and released. Ultimately, glucocorticoids have a profound effect on various brain regions thus dramatically manifested behavioral changes (Wisłowska-Stanek et al., 2016).

The hippocampus and Amygdala mediate the HPA-axis. By acting on Hippocampal and PVN neurons, glucocorticoids employ feedback effects on the HPA-axis. In normal circumstances, glucocorticoids inhibit HPA-axis activity by itself along with amelioration of cognitive and hippocampal functioning. But chronic stress conditions result in hippocampal neuron damage especially CA3 pyramidal neurons. It also causes injury to the neurons in the granular cell layer of the dentate gyrus, dendritic branching, and the glutamate input site (Tafet & Nemeroff, 2020).

Glucocorticoids acts on multiple brain areas mainly on the hippocampus via ligand mediated transcription factors leading to enhanced glutamate, endocannabinoid production/release. It also acts on mitochondria to affect the free radical formation and reduces the free radicals scavenging mechanism in brain regions. Two major receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR), mediate the effects of glucocorticoids on the cerebral regions. Both are ligand-activated transcription factors (the ligand is a steroid), resulting in either positive or negative expression of their respective target genes. The pulsatile secretion of ACTH in blood and modulation of circadian rhythm

is maintained by activation of MR. Whereas, GR has been studied in feedback modulation of glucocorticoids during stress (McEwen & Akil, 2020).

HPA-axis is regulated by the hippocampus and Amygdala regions. Cortisol or Corticosterone (glucocorticoids) acts on the hippocampal and PVN neurons that leads to an effect on HPA-axis. Under normal physiology, a specific level of glucocorticoids is continuously released that is considered normal. The normal level of glucocorticoids is essential to inhibit HPA-axis activity via a feedback mechanism, enhance cognitive abilities, and maintain normal functioning of the hippocampus. But continuous and chronic stress results in neurodegeneration by damaging hippocampal neurons especially CA3 pyramidal neurons (J.-M. Fan et al., 2014).

Glucocorticoids acts on multiple brain areas mainly on the hippocampus via ligand- mediated transcription factors leading to enhanced glutamate, endocannabinoid production/release. It also acts on mitochondria to affect the free radical formation and reduces the free radicals scavenging mechanism in brain regions. Two major receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR), are responsible for mediating the effects of glucocorticoids on cerebral regions. Both are ligand-activated transcription factors (i.e., steroid binding modifies their transcriptional activity), resulting in either positive or negative expression of their target genes. The MR influences the circadian rhythm of basal glucocorticoid and the pulsatile secretion of ACTH into the circulation. The glucocorticoid receptor (GR) has been linked to the glucocorticoid feedback system during stress (McEwen & Akil, 2020).

In short, excess stress results in abnormal HPA-axis activation leads to overproduction of glucocorticoids, and consequently may lead to depression. Elevated Levels of glucocorticoids (Corticosterone/cortisol manifested in damage to neurons of the hippocampus leading to cognitive symptoms of depression. Furthermore, enhanced CRP level due to excessive stress manifested in

abnormal arousal, appetite, and other pleasurable activities. Moreover, rewarding stimuli and psychological memory is also affected by an enhanced level of CRF (McEwen & Akil, 2020).

2.3.2 Hypothesis regarding depression

Several well-established hypotheses regarding depression exist, including the monoamine hypothesis of depression, neurotrophic hypothesis of depression, and neuroendocrine hypothesis of depression. These hypotheses help uncover the underlying pathogenic processes involved in the development of the disease (Strawbridge et al., 2017).

2.3.2.1 Monoamine hypothesis of depression

Approximately thirty years ago, the leading theory of melancholy was developed. It has been hypothesized that a lack of monoaminergic neurotransmitters such as norepinephrine (NE), serotonin (5-HT), and dopamine (DA) causes depressive symptoms (Cosci & Chouinard, 2019). Various animal studies and therapeutic monitoring are evidence of the hypothesis in question. Antihypertensive drug ie. Reserpine produced its effect by reducing NE, 5-HT, and DA leads to a syndrome similar to depression. Moreover, the compound iproniazid (Anti-tuberculosis) produces its effect by inhibiting an enzyme monoamine oxidase (MAO) enzyme that increases NE and 5-HT levels lead to euphoria and hyperactive behavior (Korte et al., 2015).

2.3.2.2 Neurotrophic hypothesis of depression

Neurotrophic factors are proteins that regulate the survival, development, and function of neurons in the central and peripheral nervous systems. According to the neurotrophic hypothesis of depression, major depressive disorder (MDD) may be partially caused by a decrease in the support provided by neurotrophic factors. Brain-derived neurotrophic factor (BDNF) is a crucial neurotrophin that is essential for the development, maintenance, and survival of neurons in key brain circuits involved in emotion and cognition. Numerous studies have focused on BDNF and have provided evidence

suggesting that changes in BDNF function and levels are associated with depression. (Duman & Li, 2012).

2.3.2.3 Neuroendocrine hypothesis of depression

Major depressive disorder (MDD) is closely associated with disturbances in neuroendocrine activity regulation. Hormonal abnormalities, such as elevated cortisol levels, failure to suppress the release of adrenocorticotropic hormone (ACTH) during the dexamethasone suppression test, and persistently elevated corticotropin-releasing hormone levels, are frequently observed in individuals with MDD. These abnormalities arise, in part, due to the overlap between brain regions that control mood and those responsible for regulating primary neuroendocrine axes and metabolic functions. It is noteworthy that the origins of neuroendocrine deficits in MDD can be traced back to fetal development, with influences from factors such as sex. These deficits typically emerge shortly after puberty and can be further triggered by events such as pregnancy (postpartum) and menopause (Chávez-Castillo et al., 2019).

2.4 Gut brain axis

The gut-brain axis (GBA) facilitates communication in both directions between the central nervous system (CNS) and the enteric nervous system (ENS), creating a link between the affective and cognitive centers of the brain and intestinal functions. Recent scientific progress has highlighted the influence and regulation of these interactions by intestinal microbiota. The autonomic system, comprised of the sympathetic and parasympathetic components, transmits both afferent and efferent signals between the brain and the intestinal wall. The enteric, spinal, and vagal pathways transmit signals from the intestinal lumen to the CNS (Carabotti et al., 2015).

2.5 Bi-directional communication routes of Gut brain axis

The gut-brain axis is bidirectional, indicating that communication occurs in both directions between the stomach and the brain. This two-way communication is facilitated by various mechanisms. Firstly, the

gut microbiota, present in the gut, can influence the central nervous system (CNS) through a "bottom-up" approach. The microbiota can impact CNS functioning by producing metabolites and other substances that can enter the bloodstream and reach the brain. Additionally, the gut microbiota can affect the CNS through the enteric nervous system, releasing neuroactive chemicals and gut hormones that can influence brain activity (Y. Du et al., 2020) .

Conversely, the CNS also influences the gut through a "top-down" approach. The hypothalamic-pituitary-adrenal axis and the autonomic nervous system permit the brain to regulate gastrointestinal function. As an example, the brain releases norepinephrine in response to stress, which has been shown to promote the proliferation of specific harmful bacteria in the intestine (Williams et al., 2014).

2.5.1 HPA axis

The hypothalamic-pituitary-adrenal (HPA) axis is predominantly responsible for the body's physiological response to stress (Stanojevi et al., 2018). It plays a crucial function in the gut-brain axis. It is a component of the limbic system, a key brain region responsible for memory and emotional responses. This system is activated when the body is exposed to environmental stress and increased levels of pro-inflammatory cytokines. This activation causes the hypothalamus to secrete corticotropin-releasing factor (CRF), which stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH). This cascade ultimately leads to the discharge of cortisol from the adrenal glands. Cortisol, a major stress hormone, exerts its effects on numerous organs, including the brain. Consequently, the brain is able to regulate the function of cells involved in intestinal processes, including immune cells, enteric neurons, epithelial cells, smooth muscle cells, interstitial cells of Cajal, and enterochromaffin cells. Concurrently, these cells are also influenced by the gut microbiota, the significance of whose function in bidirectional communication with the brain-gut axis has only lately been explored. (2014) (Mayer, Savidge, et al.).

2.5.2 Vagus Nerve

The vagus nerve is an essential component of the parasympathetic nervous system, which regulates numerous essential biological functions, such as mood, immune response, digestion, and cardiac rhythm. The vagus nerve transmits numerous signals between the brain and the digestive system, as well as other organs. The tenth cranial nerve begins in the medulla and extends to the neck, thoracic, and abdomen. It was given the name "wanderer nerve" because of its extensive and winding course throughout the human body (Breit et al., 2018).

The vagus nerve is a vital connection between the brain and the gastrointestinal system, transmitting information about the condition of the internal organ to the brain via afferent fibers. This quality makes it a desirable target for the treatment of mental and digestive disorders. Initial evidence suggests that stimulating the vagus nerve could be an effective complementary treatment for conditions such as depression, posttraumatic stress disorder (PTSD), and inflammatory bowel disease that is resistant to treatment (Y. Liu and R. Forsythe, 2021). Treatments that increase vagal tone and decrease cytokine production target the vagus nerve in particular. These strategies are essential for fostering resilience. The activation of vagal afferent fibers in the intestines influences the monoaminergic brain systems of the brain stem. These systems play a crucial role in significant psychiatric disorders, such as mood and anxiety disorders (Breit et al., 2018).

2.6 Potential mechanisms involved in microbiota-gut-brain axis in depression

While the exact mechanism by which the microbiota-gut-brain axis affects depression is not yet fully understood, accumulating evidence suggests that various pathways involving neurons, hormones, immune responses, and metabolism significantly contribute to the communication between gut microorganisms and the brain (Grenham et al., 2011). The connection between gut microorganisms and the central nervous system plays a significant role in mood and depression disorders, facilitated by various pathways such as neuronal, endocrine, and immunological signaling processes. The liver, due

to its anatomical position and blood supply, is involved in important immunological activities. The gut microbiota influences the expression of monoamine neurotransmitters, such as 5-HT, DA, and GABA, through chemical signaling, thereby influencing the development and plasticity of brain circuits associated with mood and behavior. By modulating the function of the microbiota-gut-brain axis, it is possible to prevent or control the over activity of the HPA axis, as evidenced by an increase in cortisol, adrenocorticotropin (ACTH), and corticotropin-releasing hormone (CRH) production (Y. Du et al., 2020). The mucosal immune system (MIS) serves as the first line of defense in the immunological pathway, protecting against infections, preventing the entrance of foreign antigens, inducing oral tolerance to a variety of antigens, and maintaining mucosal homeostasis. The liver, which receives microbes from the gastrointestinal tract and their metabolites via the portal vein, plays a vital role in mounting an effective innate immune response. By modulating the levels of anti- and pro-inflammatory cytokines such as IL-6, IL-1, and TNF-, alterations in gut microbiota can alleviate depressive symptoms. Enhanced expression of BDNF (brain-derived neurotrophic factor) has the potential to influence the development of depressive-like behavior in response to alterations in gastrointestinal microbiota. Collectively, these mechanisms contribute to the pathophysiology of depression (Y. Du et al., 2020).

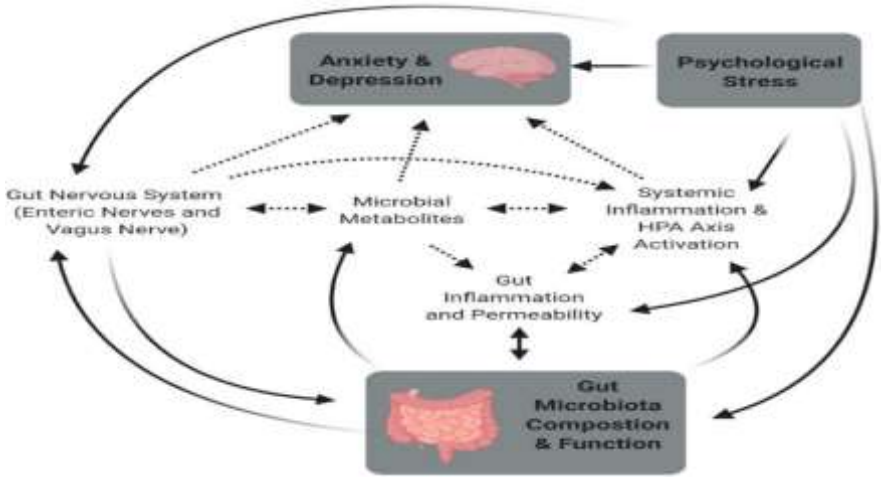

Figure 1: The microbiome gut-brain axis and stress resistance to anxiety or depression

2.6.1 Gut microbiota modulate the release and efficacy of monoamine neurotransmitters

The depletion of monoamine neurotransmitters is regarded as a major contributor to depression, and the majority of antidepressants seek to increase their concentrations in the synaptic space. Serotonin (5-hydroxytryptamine; 5-HT), dopamine (DA), and gamma-aminobutyric acid (GABA) are the three most important monoamine neurotransmitters. These neurotransmitters regulate homeostasis in the body and influence the development and plasticity of brain circuits implicated in mood disorders such as depression (Mittal et al., 2017).

2.6.1.1 Serotonin

The neurotransmitter 5-HT, which is produced by enterochromaffin (EC) cells and a network of long descending myenteric interneurons, is closely linked to a number of central neuronal mechanisms that regulate mood (Yano et al., 2015). The serotonergic system in the brain is recognized as a major biological factor in the development of mood disorders. The intestine is responsible for producing over 90% of the body's serotonin (5-HT), which is essential for gastrointestinal function and the enteric nervous system (ENS) activation (Gershon & Tack, 2007). Tryptophan decarboxylases within the intestinal microbial community convert tryptophan to tryptophan amine, thereby enhancing the ability of microorganisms to utilize dietary tryptophan. This process modulates mood and behavior by decreasing serotonin (5-HT) production in the brain (Williams et al., 2014). However, the precise molecular mechanisms involved in the regulation of serotonin (5-HT) metabolism in the colon remain obscure (Yano et al., 2015).

2.6.1.2 Dopamine

Dopamine (DA) and the dopaminergic system play a critical role in regulating anhedonia, a key symptom of major depressive disorder (MDD) (Belujon & Grace, 2017). Depressive individuals exhibit

lower dopamine transporter (DAT) binding and reduced dopaminergic activity in the striatum compared to healthy individuals. PET imaging studies have revealed that these changes are associated with anhedonia, a symptom commonly observed in depression (Pruessner et al., 2004). Dopamine (DA) not only influences intestinal motility and secretion, but also plays a role in the microbiota-gut-brain axis's functioning *E. faecium* has been demonstrated to modulate the immune system and influence the host via dopaminergic pathways. Moreover, studies involving mice treated with *Bifidobacterium* and *Limosilactobacillus* over an extended period have demonstrated increased levels of both dopamine and serotonin (5-HT), along with improved depressive-like behaviors. (Villageliú & Lyte, 2018) (Kennedy et al., 2017b).

2.6.1.3 GABA

GABA, the principal inhibitory neurotransmitter in the brain, is essential for a vast array of physiological and psychological processes. Multiple neuropsychiatric disorders, including depression, have been linked to the dysregulation of the GABA system (Kumar et al., 2013). Strains of *Limosilactobacillus brevis* and *Bifidobacterium dentium* that are known to produce GABA efficiently metabolize monosodium glutamate (MSG) to produce GABA. This suggests that modulating the microbiota may be a viable treatment for depression (Barrett et al., 2012). According to studies, the levels of *Bifidobacterium* and *Limosilactobacillus* species in the gastrointestinal tract of depressed individuals are significantly reduced. Probiotics have been proposed as a possible factor in the decreased mRNA expression of GABA receptors in depressive disorders (Bravo et al., 2011b) (Ait-Belgnaoui et al., 2014). The findings suggest that monoamine neurotransmitters may play a role in the functioning of the microbiota-gut-brain axis, which functions as a communication connection between the brain and intestinal microecology.

2.7 Conventional treatments for depression

The table depicts different treatment options, highlighting their respective mechanisms of action and profiles of side effects.

Table 1: Conventional treatment for depression

Drug Class	Drug Name	MAO	ADR's	Reference
Selective Serotonin Reuptake Inhibitors (SSRIs)	Fluoxetine, Paroxetine, Escitalopram, Sertraline, Citalopram, Fluvoxamine	Inhibits reuptake mainly of serotonin by binding to serotonin transporters.	Dry eye, decreased accommodation, and visual blurring (mainly with Paroxetine), mydriasis, precipitation of AACG, Ocular dystonia (rare), optic neuropathy (rare), and maculopathy.	(Ferguson, 2001)
Serotonin, Noradrenalin Reuptake Inhibitors (SNRIs)	Duloxetine, Venlafaxine, Desvenlafaxine, Milnacipran, Levomilnacipran	Inhibit reuptake of both Serotonin (5HT) and Noradrenaline (NA) by acting on SERT and NAT.	Mydriasis precipitation of AACG (lesser than SSRI's and TCA's)	(SNRIs) (Gałecki et al., 2018)
Tricyclic Antidepressants (TCA)	Amitriptyline, Nortriptyline, Imipramine, Desipramine, Clomipramine, Nortriptyline, Doxepin	Inhibits reuptake of both 5HT and NA by acting on SERT and NAT, Anti H1, H2 histaminic receptors, Anticholinergic	Dry eye, decreased accommodation and visual blurring (1/3rd patients), mydriasis, precipitation of AACG	(Hawton et al., 2010)
Mono Amine Oxidase Inhibitors (MAOI)	Phenelzine, Selegiline, Moclobemide	Inhibits the activity of MAO enzyme	Mydriasis and AACG precipitation	(Sub Laban & Saadabadi, 2023)

Atypical Antidepressants	Bupropion, Nefazodone, Vortioxetine, Trazodone, Mirtazapine	Dopamine reuptake Inhibitor, Serotonin receptor modulators, and reuptake inhibitors with added anti a_1 and anti H1 receptor action	Retinopathy (rare), mydriasis, and AACG precipitation (rare)	(Rahman, 2018)

2.7.1 Limitations of conventional treatments of depression

Over the past few decades, there has been a notable expansion in the range of pharmacological treatments for depression, thanks to the development of new medications and combination therapies. Imipramine, introduced by Kuhn in the 1950s, marked the beginning of this trend, leading to a substantial increase in the availability of antidepressant drugs. Tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs) effectively alleviate depressive symptoms by increasing serotonin (5-HT) and/or norepinephrine (NE) levels in the brain (Abdallah et al., 2016). Despite the fact that both tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs) are highly effective at treating depression, their use is hampered by a variety of undesirable adverse effects. Multiple transmitter systems in the brain and periphery are affected by TCAs, resulting in sedation, hypotension, impaired vision, and parched mouth, among other side effects. In cases of overdose, TCAs can be life-threatening and fatal, primarily due to their effects on the cardiovascular system. Irreversible MAOIs also pose challenges, such as the risk of dangerous hypertension when combined with tyramine-rich foods (known as the "cheese effect"). Many patients fail to adhere to suitable dosages for an adequate duration, resulting in under treatment and less severe side effects. An ideal antidepressant should possess qualities such as cost-effectiveness, safety, ease of administration, low risk of overdose,

minimal adverse effects, rapid onset of action, high efficacy rates, and minimal drug interactions (Herraiz & Guillén, 2018).

2.8 Alternative Treatment for depression

Due to certain side effects of conventional antidepressants it's high time to look for alternative treatment that are better, safe and effective or at least equal to the available treatment with no side effect. As probiotics and polyphenols has antidepressant effects and meet this demand so they can be used as a novel and effective treatment strategy against depression.

2.8.1 Probiotics

Probiotics are living microorganisms that, when consumed in sufficient quantities, can provide health advantages. These beneficial bacteria and yeasts are naturally present in certain fermented foods like yogurt, kefir, sauerkraut, kimchi, and kombucha, and they are also available as dietary supplements. Probiotics have been studied extensively for their potential to improve digestion, boost immune function, alleviate allergies, prevent infections, and even improve mental health (Hill et al., 2014b).

One of the most well-known benefits of probiotics is their ability to support gut health. The gut is home to trillions of bacteria, some of which are beneficial and some of which can cause harm if their populations get out of balance. Probiotics contribute to the rebalancing of the intestinal microbiota by encouraging the development of beneficial bacteria while inhibiting the growth of harmful bacteria. This can result in alleviation of digestive issues like bloating, constipation, and diarrhea. (Y. Liu et al., 2018).

Emerging research suggests that probiotics may have positive mental health effects. It is believed that the gut-brain axis, which facilitates communication between the intestines and the brain, has important implications for mental health. Preliminary studies suggest that probiotics could potentially alleviate

symptoms of anxiety and depression. However, further extensive research is required to comprehensively comprehend the intricacies of this relationship.(Huang et al., 2016).

2.8.1.1 Probiotics and gut microbiota

The gut microbiota, also known as gut flora, is a diverse community of microorganisms residing in the digestive tract. Important functions such as metabolism, immunity, and digestion rely heavily on it. Several health conditions, including inflammatory bowel disease, obesity, and depression, have been linked to imbalances or alterations in the composition of the intestinal microbiota. Hence, probiotics offer a means to rebalance the gut microbiota and restore its equilibrium. (Y. Fan & Pedersen, 2021)

Probiotics are beneficial live microorganisms that, when consumed in sufficient quantities, provide health advantages. They can be obtained from fermented foods or taken as dietary supplements.

The most common probiotic bacteria belong to the *Limosilactobacillus* and *Bifidobacterium* genera (Allen et al., 2016).

Numerous studies have investigated the effect of probiotics on the intestinal microbiota composition. The consumption of probiotic yogurt containing *Limosilactobacillus rhamnosus GG* was found to induce alterations in the gastrointestinal microbiota in a randomized controlled trial involving healthy adults. Specifically, it increased the levels of beneficial bacteria like *Limosilactobacillus* and *Bifidobacterium* while reducing the abundance of potentially harmful bacteria such as *Enterobacteriaceae* (Korpela et al., 2016).

2.8.1.2 Role of probiotics in reducing depression

Depression, a prevalent mental disorder, is characterized by constant sadness and a lack of interest in engaging in activities. Emerging evidence suggests that gut microbiota dysbiosis may play a role in the development of depression, although the precise causes of depression remain unknown (Capuco et al., 2020).

The gut-brain axis is a two-way network of communication between the gastrointestinal microbiota and the central nervous system. Through this axis, the intestinal microbiota can influence the production of neurotransmitters, such as serotonin, which play an essential role in modulating mood and behavior. Disruptions in the gut microbiota can impact the synthesis and availability of neurotransmitters, potentially resulting in alterations in mood and behavior (Winter et al., 2018).

Numerous studies have examined the potential effects of probiotics on depression. Ng et al. conducted a meta-analysis that included ten randomized controlled trials examining the effects of probiotics on depressive symptoms. The meta-analysis found that probiotics significantly reduced depressive symptoms compared to placebo. The effect size was small to moderate, but the authors concluded that probiotics may be a useful adjunctive therapy for depression (Korpela et al., 2016).

Research has also shown that probiotics can increase neurotransmitter levels in the brain, which may explain their positive effects on mood. Neurotransmitters are chemical mediators in the brain that, among other functions, serve a crucial role in regulating mood. Low concentrations of neurotransmitters such as serotonin and dopamine are associated with depression and anxiety. Probiotics have been shown to increase the production of these neurotransmitters, which can improve mood and reduce symptoms of depression (J. Kelly et al., 2015).

Serotonin is a neurotransmitter that has significant role in the regulation of mood, appetite, sleep, and other important functions. Approximately 90% of serotonin is synthesized in the gut, and alterations in gut microbiota composition can affect serotonin production (Chen et al., 2021). Probiotics may modulate serotonin production through several mechanisms. One mechanism is the production of the essential amino acid tryptophan, which is a precursor to serotonin. *Limosilactobacillus* and *Bifidobacterium* are two probiotic bacteria that are known to produce tryptophan (O'Mahony et al., 2015).

Probiotics may also influence serotonin production by modulating the expression of the rate-limiting enzyme in serotonin synthesis, tryptophan hydroxylase. A study found that the consumption of a

probiotic mixture containing *Limosilactobacillus rhamnosus, Bifidobacterium bifidum,* and *Limosilactobacillus helveticus* increased tryptophan hydroxylase expression in the rat brainstem (Desbonnet et al., 2010) (O'Mahony et al., 2015).

2.8.1.3 *Limosilactobacillus* Genera

Limosilactobacillus fermentum type of lactic acid bacteria, has garnered considerable attention for its potential health benefits. One particular area of interest lies in its potential impact on mental health, specifically in alleviating symptoms associated with depression and anxiety (Del Toro-Barbosa et al., 2020).

Studies have shown that *Limosilactobacillus fermentum* may have a positive impact on mood and anxiety levels. In one study, participants who consumed a probiotic containing *Limosilactobacillus fermentum* experienced a significant reduction in symptoms of anxiety and depression compared to a placebo group (Misra & Mohanty, 2019).

Another research study demonstrated that the supplementation of *Limosilactobacillus fermentum* resulted in enhanced cognitive function and a reduction in symptoms related to depression and anxiety among individuals with chronic fatigue syndrome (Mohajeri et al., 2018). The findings indicate that *Limosilactobacillus fermentum* could potentially have beneficial effects on mental health and cognitive function.

Although the precise mechanism through which *Limosilactobacillus fermentum* exerts its beneficial effects on mental health has yet to be completely elucidated, it is believed that the bacteria may affect the gut-brain axis. This axis is a pathway for communication between the gastrointestinal system and the central nervous system (CNS) (Cryan et al., 2019). The gut-brain axis has been linked to the modulation of mood and behavior, and disturbances in the composition of gut microbiota have been linked to mental health disorders (Foster et al., 2017).

In conclusion, the research indicates that *Limosilactobacillus fermentum* holds promising potential as a natural intervention for enhancing mental health and alleviating symptoms of depression and anxiety. However, further investigation is necessary to comprehensively comprehend its mechanisms of action and determine the most effective dosages and delivery methods.

2.8.2 Polyphenols

Polyphenols, a class of naturally occurring compounds derived from plants, have the potential to treat a variety of neuropsychiatric disorders, including depression (Donoso et al., 2020) (Dhir, 2017). Polyphenols have been demonstrated to impact multiple pathways associated with the development of depression. They influence diverse neurotransmitter systems, the hypothalamic-pituitary-adrenal (HPA) axis, and intracellular signaling pathways involved in neurogenesis, neuroplasticity, and cell survival (Zhou et al., 2020) .

Polyphenols, abundantly present in various plant sources, are associated with numerous health benefits, including potential antidepressant effects. While some polyphenols can be absorbed in the small intestine, due to their complex structures, the majority have limited bioavailability and reach the large intestine unaltered. The interaction between polyphenols and gastrointestinal microbial populations is crucial for releasing polyphenols' antidepressant potential (Zhou et al., 2020). The gut microbiota serves a crucial role in increasing the bioavailability of polyphenols, while polyphenols contribute to sustaining the intestinal barrier and the gut microbiota population. Furthermore, gut microbes metabolize polyphenols into metabolites that are more potent and easily absorbed, thereby exerting their antidepressant effects through the microbial-gut-brain (MGB) axis (Zhou et al., 2020).

In addition, the polyphenols found in fruits, vegetables, tea, and chocolate have been found to influence the function and expression of key signaling molecules involved in inflammatory processes related to depression. This suggests that they hold significant potential in the development of novel antidepressant medications targeting inflammation-related depression (Caracci et al., 2020). Recent research indicates

that plant-derived sources and their bioactive phytochemicals have therapeutic effects on mental disorders, offering a broad spectrum of efficacy with minimal adverse effects (Ko et al., 2020). Consuming polyphenol-rich foods is a non-invasive, natural, and cost-effective way to improve brain health. The ability of polyphenols to stimulate the production of nitric oxide (NO) in the endothelium and reduce platelet aggregation not only increases cerebral blood flow but also has neuroprotective effects. (Caracci et al., 2020) (Gomez-Pinilla & Nguyen, 2012).

2.8.2.2 Chrysin

Chrysin, a naturally occurring flavonoid (C15H10O4, molecular weight: 254.24 g/mol), has garnered significant attention due to its diverse pharmacological properties (S. Gao et al., 2021). Belonging to the subgroup of flavones, chrysin possesses a 15-carbon structure and is predominantly found in honey and propolis (Naz et al., 2019). Chrysin exerts its pharmacological effects through the regulation of various signaling pathways and modulation of multiple molecular targets. As a result, it exhibits diverse properties, including anti-inflammatory activity. (Siddiqui et al., 2018). Extensive research has been conducted on chrysin's anti-inflammatory properties. It has been discovered to inhibit the expression of genes that produce inflammatory molecules, such as pro-inflammatory cytokines, cyclooxygenase, and iNOS. This effect is accomplished by inhibiting critical inflammatory signaling pathways including NF-B, MAPK, and STAT (Adangale & Wairkar, 2022) (Zeinali et al., 2017). Moreover, chrysin exhibits notable neuroprotective and neurotrophic properties, making it a potential therapeutic agent for addressing cognitive impairments (Nabavi et al., 2015b). Recent studies on animals have shown that chrysin can ameliorate behavioral symptoms associated with emotional disorders (Ko et al., 2020). The neurochemical mechanisms underlying the antidepressant properties of chrysin were investigated in rodents undergoing OBX (olfactory bulbectomy) surgery. The OBX-treated animals exhibited depressive-like behaviors, decreased levels of brain-derived neurotrophic factor (BDNF), and elevated levels of pro-inflammatory cytokines including IL-1, IL-6, TNF-, and IFN. Enhanced IDO (indoleamine

2,3-dioxygenase) activity in the hippocampus region was responsible for the dysregulation of the TRP-KYN pathway (Del Fabbro, Donato, Gomes de Gomes, et al., 2016a). In the hippocampus of OBX animals with hyperactive IDO, levels of serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) were reduced and increased, respectively. However, administration of chrysin to OBX rodents prevented these neurochemical changes and ameliorated their depressive-like behavior. Chrysin effectively alleviated depressive symptoms in rats exposed to prolonged and unpredictable stress. In a study, antidepressant-like effects of chrysin were associated with decreased levels of pro-inflammatory cytokines and kynurenine (KYN), downregulation of IDO, caspase-3, and caspase-9 activity, increased levels of 5-HT in the prefrontal cortex (PFC) and hippocampus, and decreased plasma levels of ACTH and CRH (Filho, Jesse, Donato, Del Fabbro, de Gomes, et al., 2016). These results suggest that the anti-inflammatory properties of chrysin are associated with its effect on the TRP-KYN pathway and 5-HT levels, which ultimately contribute to the amelioration of depression-like behavioral symptoms observed in chrysin-treated rodents. By regulating inflammatory mediators and mitigating oxidative and apoptotic damage, chrysin exerts antidepressant-like effects on clonidine-induced depressive-like behavioral despair in rodents, according to a recent study. Moreover, chrysin ameliorates behavioral and cognitive deficits associated with traumatic brain injury (TBI) in rodents, possibly by inhibiting neuroinflammation and preventing neuronal loss in the cerebral cortex and hippocampus (Tayab et al., 2022c).

Overall, chrysin and other polyphenols have the potential to be used as natural antidepressants and anxiolytics, without the adverse effects associated with conventional drugs.

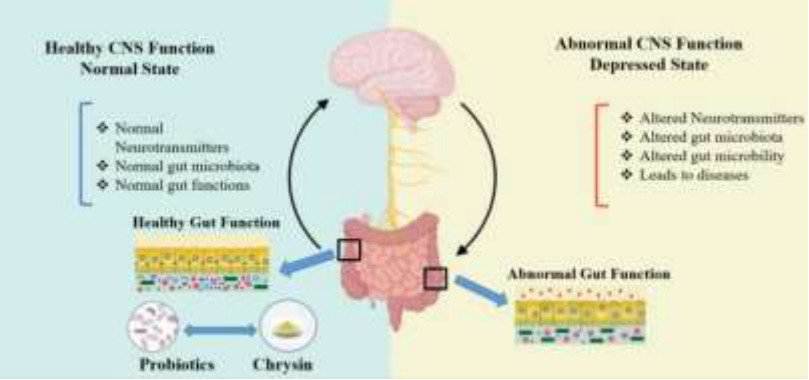

Figure 2: Mechanism of action of Probiotic and Chrysin in depression

CHAPTER 3

3.0 Materials and methods

3.1 Chemicals and reagents:

Chrysin powder ($C_{15}H_{10}O_4$) was purchased from Sigma Aldrich (CAS-No: 480-40-0). Fluoxetine was bought from local pharmacy in Islamabad, Pakistan. MRS Broth and MRS agar were got from MERK. Chemicals for gram staining like Safranine, Gram's Iodine, Crystal Violet and Decolorizer were brought from Diachem.

3.2 Probiotic selection (*Limosilactobacillus fermentum*)

The probiotics used were a mixture of three strains of *Limosilactobacillus fermentum* (7b, 18, 19b) which were previously isolated by my lab senior, Muneera Ahmad in 2016.

3.2.1 Morphology assessment of probiotic strains (*L. fermentum)*

For morphology assessment of probiotic strains (*Limosilactobacillus fermentum* 7b, 18, 19b) gram staining and catalase test was done. A single colony was carefully selected from the MRS agar plate and placed on a slide containing a droplet of distilled water. The smear was heat-fixed and subsequently treated with crystal violet for 1 minute, followed by rinsing with distilled water. Next, it was treated with an iodine solution for 40 seconds, washed briefly with ethanol for 5 seconds, and stained with safranin for 40 seconds. After a final rinse with distilled water, the slide was allowed to air dry and examined using immersion oil under a 100X objective lens microscope. Subsequently, a catalase test was performed by transferring a single colony of the probiotic strain onto a clean, dry slide using a sterile loop. 3% H2O2 was added to the colony and thoroughly mixed.

3.3 Probiotic dosage:

Probiotic dosage of 1 x 10^9CFU/ml/day was given to 2 groups of mice. Plate count technique was used to enumerate the number of cells in the dose. Dosage was administered through drinking water to mice. Dose was prepared on daily routine, overnight fresh culture was centrifuged at 6000rpm for 10mins at 4°C. Supernatant was discarded and bacterial cell pellet was washed with PBS two to three times. After that, compared the dosage turbidity with 0.5 McFarland standard. Optical density of dose was checked and matched with 0.5 McFarland standard i.e. 0.08-0.1. Fresh dosage was prepared daily for administration of dosage to mice.

3.4 Animals:

36 Balb/c mice of age 6-8 weeks and male gender, weighing 25-30g 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.

3.4.1 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-07-2022-ASAB-01/01 approved the present study.

3.5 Study design

Prior to initiating animal trials, probiotics were retrieved from the stocks and subjected to gram staining and catalase testing. Following this, a Chronic Unpredictable Mild Stress (CUMS) mice model was developed over a span of 28 days. All treatment groups received a daily dosage of probiotics at 1 x 109 CFU/ml/day and chrysin at a dose of 25mg/kg. The control groups, both normal and diseased, did not receive any treatment. A one-month plan was devised to assess the impact of probiotics and chrysin on

the CUMS mice model of depression and subsequently compare their effects both individually and in combination with the CUMS and positive control (fluoxetine) models. Subsequently, behavioral tests were conducted to evaluate anxiety, depression, and memory, followed by the decapitation of animals for histopathological studies.

3.5.1 Development of CUMS mouse model

For screening of probiotic and chrysin potential to ameliorate the progression and severity of depression, CUMS (Chronic Unpredictable Mild Stress) model with different stressors was developed. The CUMS protocol comprised of many stressful periods planned throughout the week for a duration of 28 days. In one week, these stressors were applied in a random order without recurrence. Over the four-week stress period, each stressor was repeated two to three times. The mice in the negative control group were free from any stress or treatment (Li et al., 2019a).

Table 2: Weekly schedule of the chronic unpredictable mild stress (CUMS) protocol

Mon	45° cage tilt (12 hours, hard to get food and water)
Tues	Tail pinching (60 s, 1 cm from the end of the tail)
Wed	Swimming in 4°C cold water for 5 minutes (using plastic drum-the water depth was determined by the mice toes reaching the bottom of the container)
Thurs	reversed light/dark cycle for 24h
Fri	Restraint stress (animals were placed in 50ml falcons tube to restraint their movement)
Sat	Damp bedding for 15 hours
Sun	Cage shaking for 5 minutes

Animals were randomly distributed into 6 groups: negative control, positive control, diseased group, probiotic treatment group, chrysin treatment group and probiotic+chrysin treatment group, such that

each group comprised of 6 subjects (n=6). Total number of mice is n=36. Negative control mice were provided with distilled water, positive control mice received 18mg/kg of fluoxetine +CUMS, diseased group received CUMS only, probiotics treatment group received probiotics (3 strains of *Limosilactobacillus fermentum* in a consortium) +CUMS, chrysin group received chrysin (25mg/kg) +CUMS and probiotics + chrysin group received both probiotics and chrysin +CUMS according to pre-established protocols. Chrysin was given by oral gavage by using 1.5-inch, 20-gauge stainless steel feeding needles with a 2.25 mm ball.

Table 3: Experimental groups used for this study

Sr. No	**Experimental Groups**	**(n)**	**Treatment**
1	Negative Control	6	Distilled water only
2	Positive Control	6	Fluoxetine 12mg/kg + CUMS
3	Diseased Group	6	CUMS
4	Probiotic Treatment Group	6	Probiotics 1*109 CFUs+ CUMS
5	Chrysin Treatment Group	6	Chrysin 25mg/kg + CUMS
6	Probiotic + Chrysin Treatment Group	6	Probiotics 1*109 CFU+ Chrysin 25mg/kg+ CUMS

3.5.2 Behavioral tests:

To reduce variability caused by circadian rhythms, behavioral tests were administered between 9 a.m. and 6 p.m., during the mouse light cycle. The testing chamber was maintained at a temperature of 22±2°C and was well-lit to minimize environmental disturbances and human interference. Tests were separated by 30 minutes gap for greater accuracy.

3.5.2.1 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 according to a predetermined protocol by (Komada et al., 2008). The apparatus comprised of 4 arms of which 2 were enclosed alleys and 2 were open alleys. The apparatus was constructed from an opaque iron alloy, was elevated 75.5cm from the ground, and had 30 x 5cm arms for each arm. Each mouse was situated at the intersection of the maze facing away from the experimenter and towards one of the two enclosed arms for a single 5-minute trial. A video was recorded for behavior analysis focusing on (a) the total number of entrances into EPM's open arms and (b) the total duration spent in both open arms. Sprays of 70% ethanol were used to sanitize the apparatus between trials to prevent behavioral changes due to olfactory cues.

3.5.2.2 Open field test:

This laboratory test is designed to measure the locomotor activity and anxiety levels of mice (Farhat et al., 2017). To observe exploratory behavior, the animal was placed in a square-shaped arena with dimensions of 40x40x40cm for thirty minutes. A camera aided in monitoring their movement through recording time spent within both central and peripheral regions throughout this duration as well as number of rearing incidents that serve as an indication for exploration (Farhat et al., 2017). Anxiety can be determined based on amount grooming displayed by rodents during relaxed or anxious states. This will help set baselines such it easier to detect any abnormal changes over time.

3.5.2.3 Forced swim test:

The forced swim test is used to assess depressive behaviors in rats and mice. The animals are subjected to stress by placing them in water container, making their escape impossible. This situation leads to the development of tendency of depression (Yankelevitch-Yahav et al., 2015). A few modifications were made to the previously described (Abel et al., 1990) test protocol.

For the experiment, a transparent container with a height of 50 cm and a diameter of 20 cm was filled with water at 25 2°C. The water profundity was adjusted based on the size of each mouse, ensuring that their hind legs and tails did not contact the bottom. Once placed into containers, mice were filmed using a camera recording their activities over 6 minutes after which time they were removed from it and another mouse tested if necessary by again filling up fresh warm water in emptied out container. Upon completion, latency to immobility (time taken by mice before freezing), amount spent while immobilized as well total number episodes of complete or partial paralysis experienced throughout trial period were all noted down meticulously for later analysis.

3.5.2.4 Sucrose preference test

The sucrose preference test is frequently used to assess anhedonia, a fundamental symptom of depression. The Sucrose preference test was administered immediately following the Chronic Unpredictable Mild Stress (CUMS) protocol. The rodents were exposed to two flasks of 1% sucrose solution for 24 hours to acclimate them to the solution. After 24 hours, they were exposed to one bottle of sucrose solution and one bottle of water (Li et al., 2019b). After 12 hours without sustenance or water, a 12-hour sucrose preference test was administered. During the experiment, rodents were individually housed and had access to two bottles: one containing 1% sucrose solution and the other containing water. After six hours, the positions of the bottles within the enclosures were reversed in an effort to minimize any potential side-preference effects. By weighing the vessels, the quantity of sucrose solution, water, and total liquid consumption was determined. The sucrose preference was computed as the percentage of sucrose solution ingested relative to the total volume of liquid consumed (Li et al., 2019b).

Preference value (percent) = sucrose intake/ (sucrose intake plus water intake) 100 percent.

3.5.2.5 Morris water maze test

MWM is an important test that tests hippocampal-dependent learning and long-term spatial memory. It includes a five-day acquisition phase followed by a one-day exploratory trial. The MWM is a circular basin of metal. There were visual signals situated in each of the four quadrants of the pool (east, west, north, and south). The MWM is comprised of a transparent platform and water (22°C ±2 ºC). The platform was positioned in the northwest sector. The duration of the acquisition phase was five days. Each day, five trials were administered with a 10-minute interval between each trial for each rodent. The rodents were carefully deposited in the pool from various directions. The directions in which they were released were according to the arrangement in Table 4. The time limit was 90 seconds. If the mouse did not reach the platform within 90 seconds, it was guided to the platform and held there for 20 seconds. If the mouse was able to locate the platform within the allotted time, it was permitted to remain on the platform for an additional five seconds before being removed. For the sixth day's probing trial, the platform was removed from the maze. A video was captured while the rodents explored the maze for 90 seconds.

Four parameters were calculated (Higaki et al., 2018).

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

Table 4: Direction of release of mice for Morris Water Maze 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	Direction of release = West (Single trial without a platform)				

3.6 Statistical analysis

Using Graphpad prism version 8.0.1, tests of One-way analysis of variance (ANOVA) or two-way ANOVA were applied to the dataset to ascertain whether statistical significance exists. Bonferroni's test for multiple comparisons was also used to identify differences between groups. Mean ± SEM was utilized to illustrate error bars.

3.7 Histological analysis of brain and colon tissues

3.7.1 Tissue perfusion and slide preparation

For histological analysis heart perfusion was performed according to (Hanafy et al., 2016). Briefly, the whole brain and colon was collected and washed with PBS before being transferred to 4% paraformaldehyde (PFA) solution for 24 hours and was swirled occasionally. The brain and colon 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.

3.7.2 Haematoxylin and Eosin staining

Standard haematoxylin-eosin (H and E) staining 5μm tissue section (using SLE Mainz microtome CUT 6062) was performed. Tissue after being deparaffinized was incubated (8 minutes) in Mayer's haematoxylin solution and for 10 minutes, washed in warm water. 95% ethanol was used to dip the sections and counterstaining was done with eosin (30s).

CHAPTER 4

4.0 Results

4.1 Phenotypic Identification:

4.1.1 Colony morphology:

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

4.1.2 Gram staining:

Gram staining was performed for all the selected isolates. All isolates were gram positive.

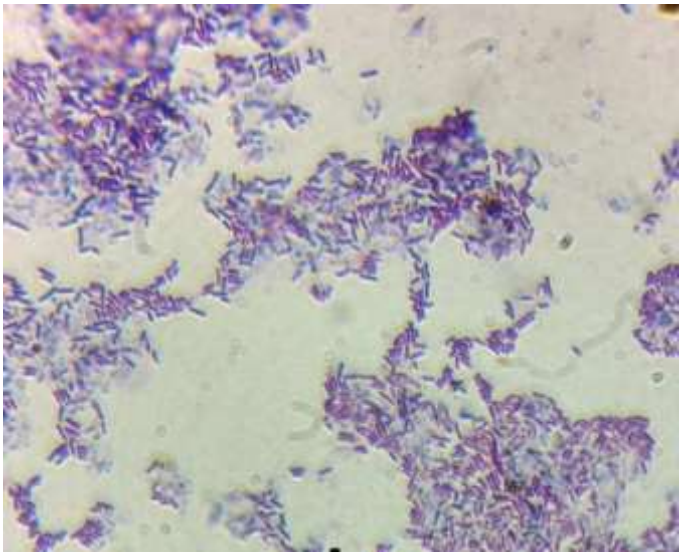

Figure 3: Different gram positive and rod shape morphologies of isolates as reveled by gram staining observed under compound microscope at 100X resolution.

4.1.3 Catalase test:

No bubble formation was observed when isolates were treated with 3% hydrogen per oxide. So, all isolates were catalase negative.

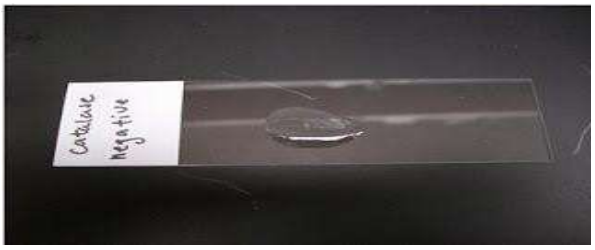

Figure 4: Catalase negative activity of isolates

4.2 Behavioral analysis to assess anxiety and depression

4.2.1 Effect of *Limosilactobacillus fermentum* (7b, 18, 19b) and Chrysin on anxiety in EPM

As a measure of anxiety, EPM was used to compare the anxiolytic effects of probiotics and chrysin to those of fluoxetine. Animals with high levels of anxiety tend to remain in closed arms, whereas animals with lower levels of anxiety are more likely to investigate the open arm. Entries in open arm in diseased control group were lower ($p<0.001$) when compared to the number of entries made by normal control. Treatment with probiotic and chrysin treated group displayed significantly higher entries into open arms ($p<0.001$) in comparison to diseased control. Treatment with probiotics treated group showed more number of entries ($p<0.01$) than diseased control. Also, positive control (fluoxetine) showed better results ($p<0.05$) when compared to diseased control.

The diseased control group showed significant differences in their time spent exploring open arms when compared to the normal controls ($p<0.001$). TPC group, treatment with probiotics and chrysin, spent significantly more time in open arms ($p<0.001$) as compared to diseased control group. TP, probiotics treated group, has significantly high performance ($p<0.01$) as compared to diseased control group. TC, treatment with chrysin, also showed good performance ($p<0.05$) as compared to diseased control group. Also, positive control group showed good results as compare to normal group ($p<0.05$). In conclusion the combination of probiotics and chrysin treated groups displayed strong anxiolytic effect in all treated

groups. This indicates that this combination of probiotics along with chrysin has strong anxiolytic properties.

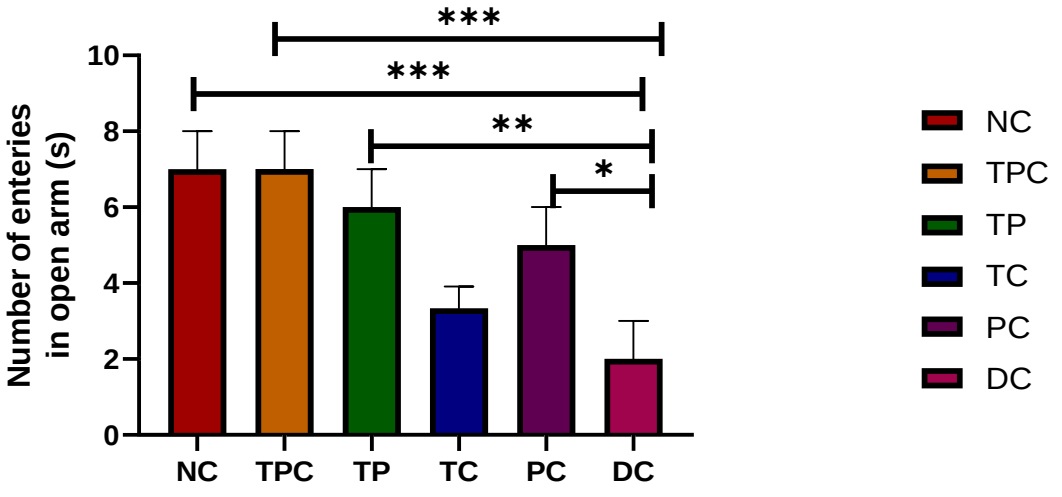

Figure 5: The effect of *L. fermentum* and chrysin on Number of enteries in Open arm in EPM

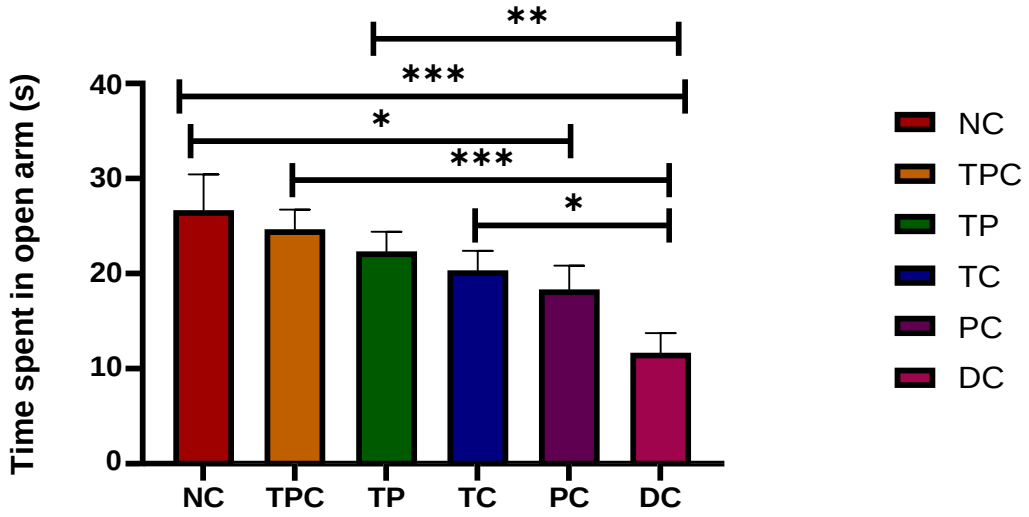

Figure 6: The effect of *L. fermentum* and chrysin on Time spent in Open arm in EPM

4.2.2 Effect of *Limosilactobacillus fermentum* (7b, 18, 19b) and Chrysin on anxiety in OFT

This test was designed to identify the anxiety and exploratory behavior of each mouse when given probiotics and chrysin versus a diseased control group. The parameters analyzed included time spent in center and at periphery, as well as number of rearings. All groups showed some level on exploration activity except for the diseased control group which had minimal amounts of exploration in centre. Diseased control spent most time at periphery and least time at center, showing significant difference ($p<0.001$) from normal control and treatment probiotic and chrysin combination, TPC, group. Diseased control showed significant difference ($p<0.01$) from treatment probiotic group and positive control (fluoxetine) in time spent at periphery as well as time spent at center. Also, significant difference was observed in treatment chrysin group ($p<0.05$) against diseased control group in time spent at periphery as well as time spent at center. Positive control showed good difference ($p<0.05$) from normal control in time spent in periphery as well as center.

No. of rearing by hind paws were analyzed to assess the exploratory behavior of animals. Normal control group followed by combination of probiotic and chrysin group showed highest number of rearing ($p<0.001$) as compared to diseased control group. Treatment probiotic group showed significant difference in number of rearings as compared to diseased control group ($p<0.05$). All groups treated with a combination of probiotics and chrysin demonstrated a potent anxiolytic effect. This indicates that this probiotic and chrysin combination has potent anxiolytic properties.

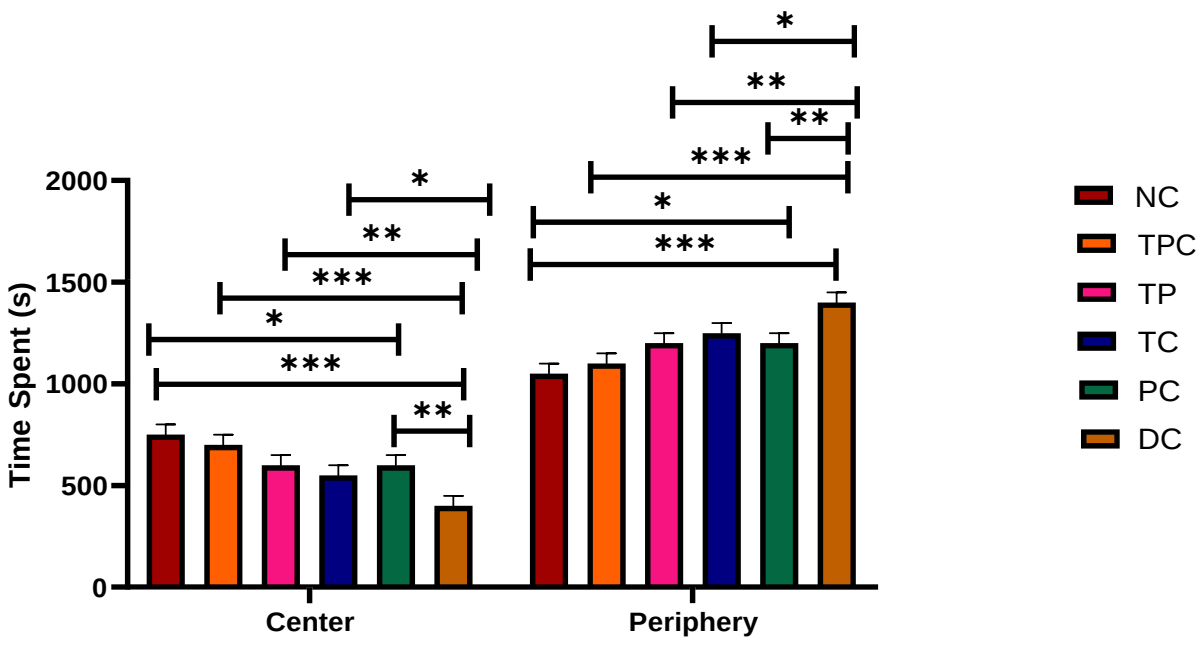

Figure 7: The effect of *L. fermentum* and chrysin on Time spent in Open arm in OFT.

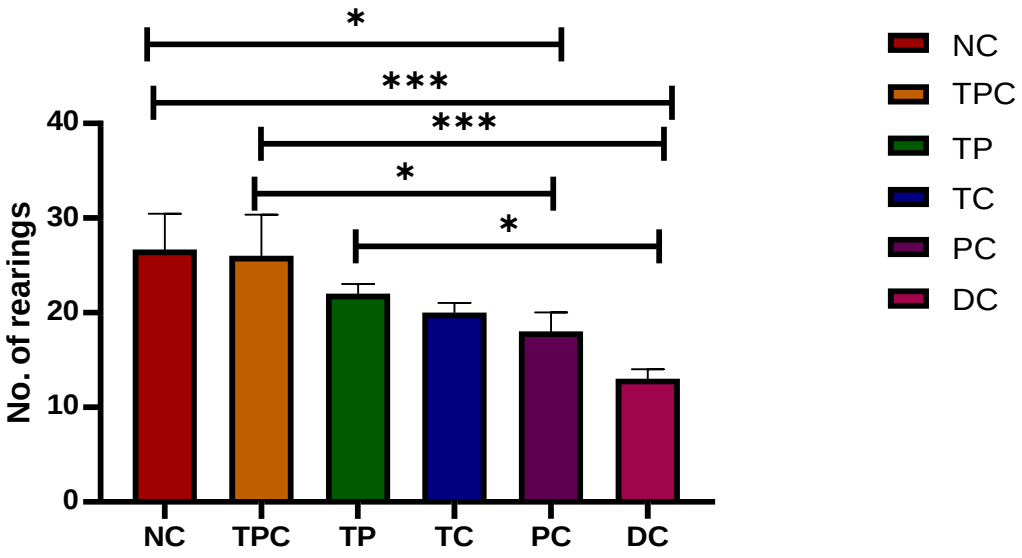

Figure 8: The effect of *L. fermentum* and chrysin on Number of rearings in Open arm in OFT.

4.2.3 Effect of *Limosilactobacillus fermentum* (7b, 18, 19b) and Chrysin on depression in FST

The forced swim test was conducted to assess the antidepressant effect of probiotics and chrysin on rodents with impaired higher cognitive functions. All groups spent significantly more time resisting, with the exception of the diseased control group, which displayed relatively greater depression and a greater number of episodes of immobility. Compared to all other groups, the number of immobile episodes was substantially higher in the diseased control group. Control showed least number of immobile episodes followed by TPC, combination of probiotic and chrysin treatment group, probiotic group, chrysin group and positive control group ($p<0.001$, $p<0.001$, $p<0.01$, $p>0.05$, $p>0.05$). Diseased control group spent more time immobile and significant difference was observed from all other groups.

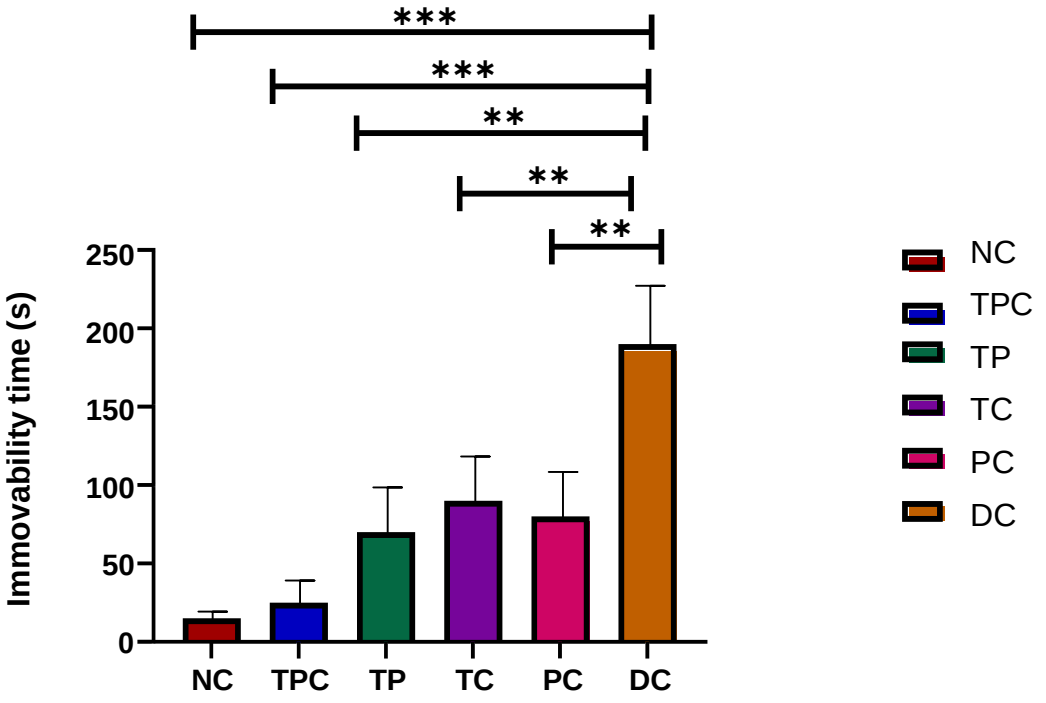

Figure 9: The effect of *L. fermentum* and chrysin on immovability time in FST.

4.2.4 Effect of *Limosilactobacillus fermentum* (7b, 18, 19b) and Chrysin on depression in SPT

The sucrose preference test, which is a reward-based test, is commonly used to evaluate anhedonia, a key symptom of depression characterized by a diminished ability to experience pleasure. Initially, rodents have a natural attraction to sugary foods and solutions. A decrease in preference for a sweet solution during the sucrose preference test, however, is indicative of anhedonia. Diseased control group clearly does not prefer sucrose water as compared to all other groups thus indicating depression. Normal group and TPC (Probiotic and chrysin) group significantly choose sucrose water over plain water ($p<0.001$) as compared to diseased group. Similarly, treatment probiotics, treatment chrysin and positive control showed significant preference to sucrose water ($p<0.01$, $p<0.01$, $p>0.05$) as compared to diseased control. Also, positive control group showed good results as compare to normal group ($p<0.05$). In conclusion, among all the groups combination of probiotics and chrysin demonstrated an antidepressant effect. This indicates that the combined use of probiotics and chrysin possesses robust properties for treating depression.

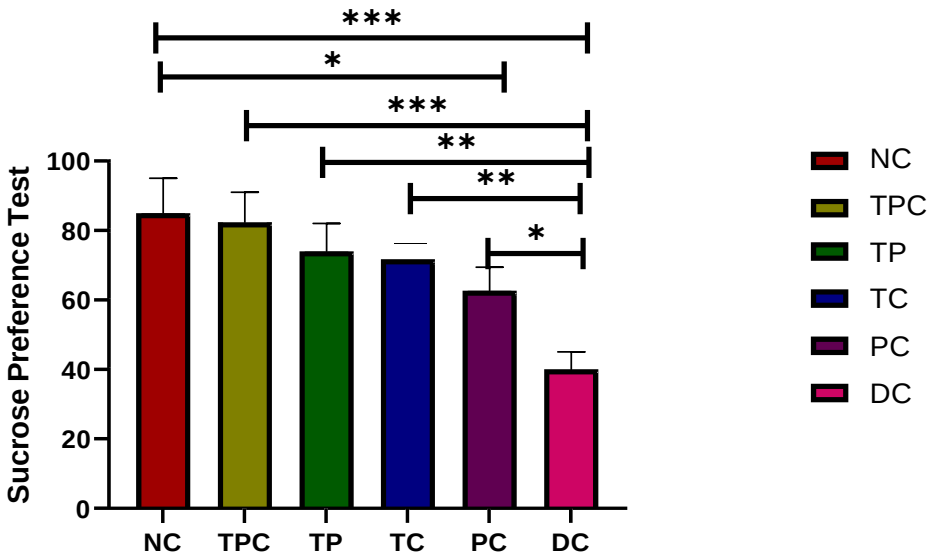

Figure 10: The effect of *L. fermentum* and chrysin on Sucrose Preference Test, SPT.

4.2.5 Effect of *Limosilactobacillus fermentum* (7b, 18, 19b) and Chrysin on spatial learning and memory in MWM

The Morris Water Maze (MWM) is a 6 day test used to measure spatial learning ability in subjects. It begins with a training phase of five consecutive days, during which time the subject's escape latency - or speed at finding cues and memorizing them - are tracked. On the sixth and final day, known as the probe trial, reference memory is measured by gauging number of crosses over platform area and amount of time spent there.

On the fifth day of the training phase, all groups except the diseased group displayed an improvement in escape latency. Diseased group displayed significantly poor ($p<0.001$) improvement in escape latency on the 5th day as compared to control group. The TPC and NC group displayed improved escape latency ($p<0.001$) as compared to diseased group.

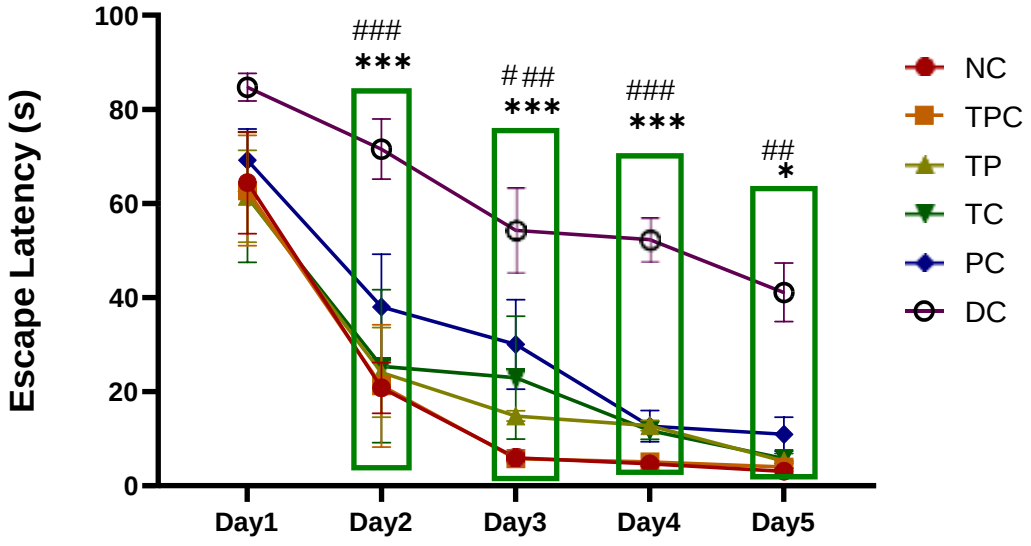

Figure 11: The effect of *Limosilactobacillus fermentum* and Chrysin on escape latency in MWM

The control group made significantly more crossings ($p<0.001$) than the diseased group. The TPC, combination of probiotic and chrysin group had significantly higher performance ($p<0.01$) than the

diseased control group. The TP, probiotic group has a significant improvement ($p<0.05$) as compared to diseased control.

Time spent in target quadrant was also evaluated. The control group followed by TPC, combination of probiotic and chrysin group, significantly spent more time in target quadrant ($p<0.001$) as compared to diseased control. Time spent in probiotics group was significantly higher in probiotics group, TP, ($p<0.01$) in comparison to diseased control. Diseased control group spent less time in target quadrant ($p<0.05$) when compared with chrysin group and positive control group.

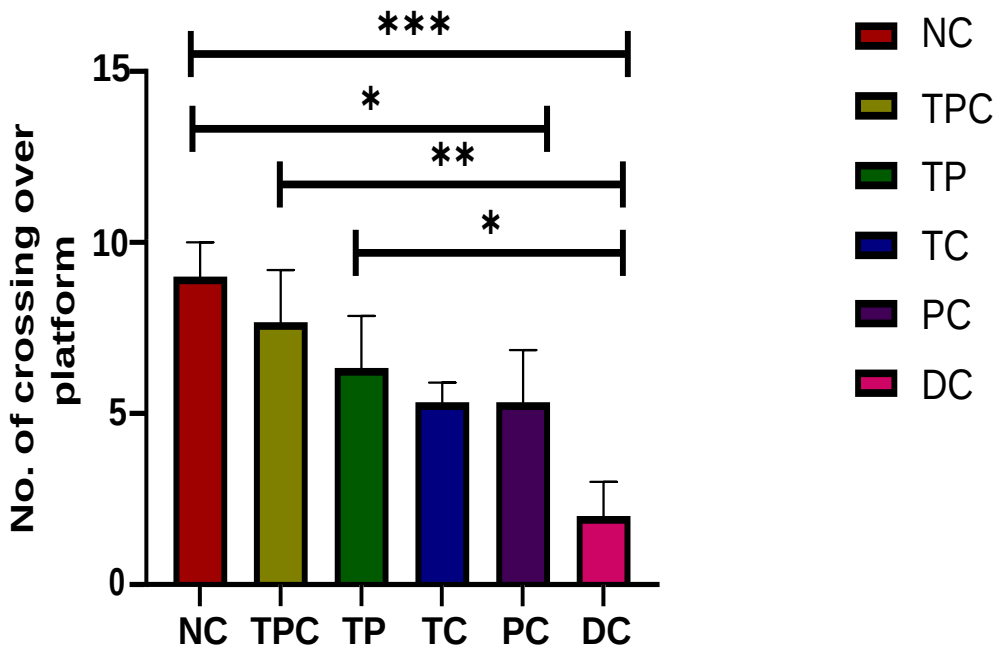

Figure 12: The effect of *L. fermentum* and chrysin on No. of crossing over platform in MWM.

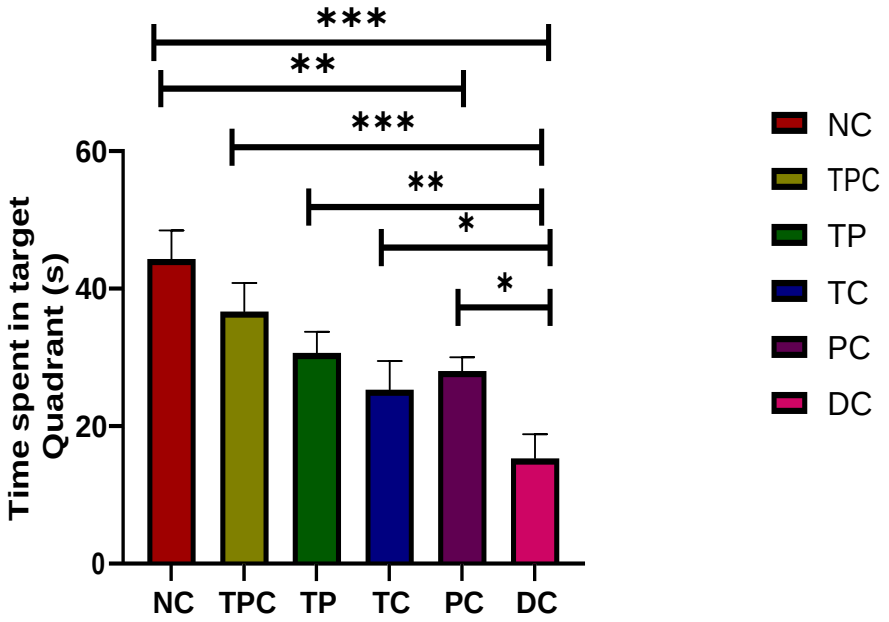

Figure 13: The effect of *L. fermentum* and chrysin on Time spent in target quadrant in MWM.

4.3 Histological examination

Histopathological analysis of all study groups was performed by H&E staining to assess morphological changes in the hippocampus and colon. The H&E staining revealed shrunken neurons, hyperchromatic nuclei and vacuolated cytoplasm in diseased control group as compared to normal, TP and TPC group. A marked difference was observed in cell bodies in diseased group as compared to normal and TPC group. A significant difference was also observed in probiotic control group as compared to diseased control. Cell counting was conducted at 10X in the dentate gyrus region of hippocampus.

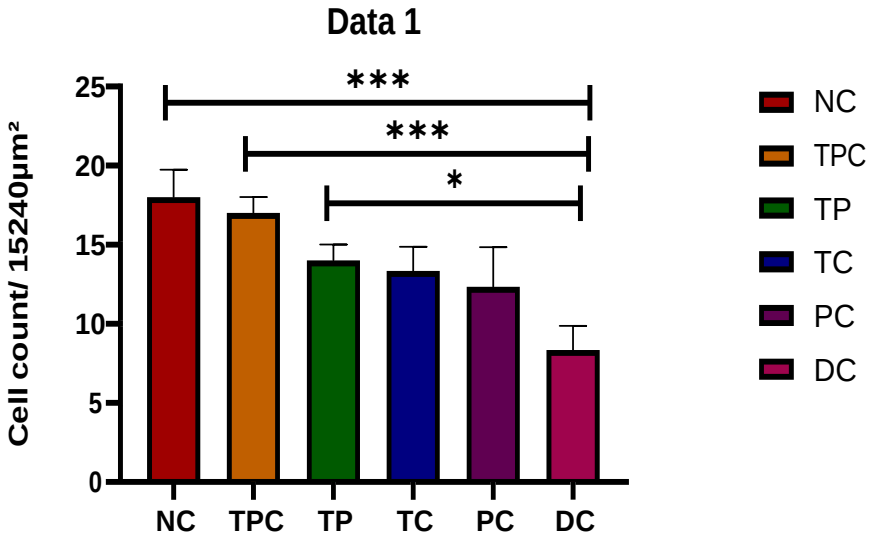

Figure 14: The effect of *Limosilactobacillus fermentum* & Chrysin and fluoxetine on cell count in dentate gyrus, hippocampus.

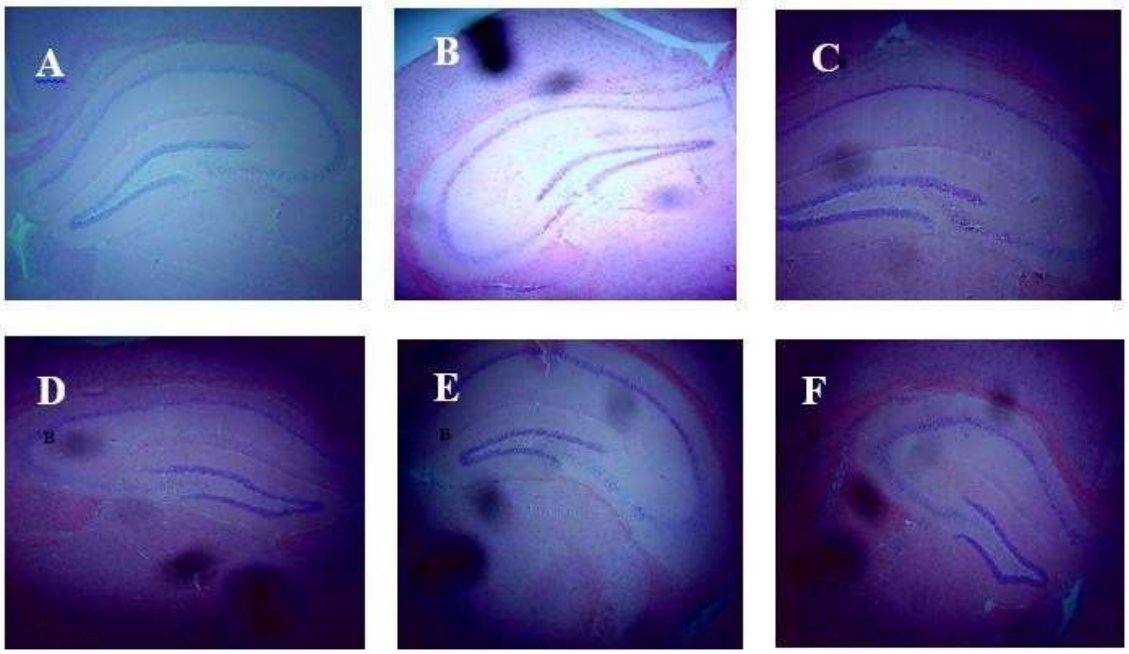

Figure 15: Haematoxylin and Eosin stained coronal sections of hippocampus 4X magnification.

(A) Control (B) Diseased Control (C) Positive Control (D) Treatment with Chrysin (E) Treatment with Probiotics (F) Treatment with Chrysin and Probiotics

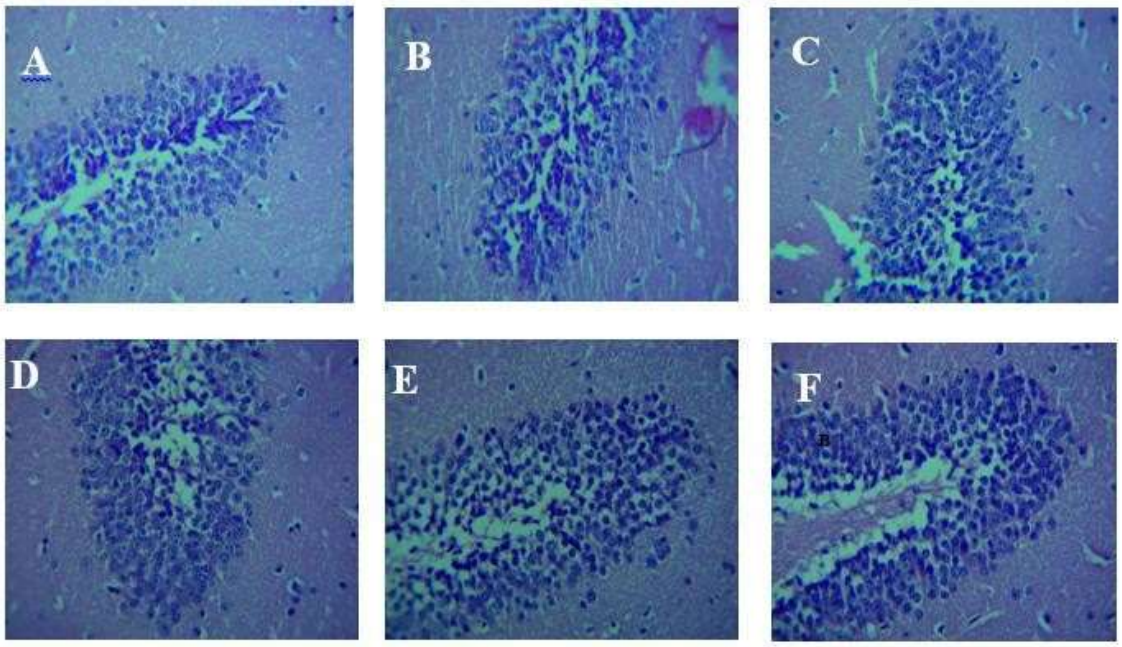

Figure 16: Haematoxylin and Eosin stained coronal sections of hippocampus 40X magnification.

(A) Control (B) Diseased Control (C) Positive Control (D) Treatment with Chrysin (E) Treatment with Probiotics (F) Treatment with Chrysin and Probiotics

In colon tissues, a loss of integrity and structure was observed in the villi. Additionally, there were signs of injury to the crypts with observable vacuolization as well as an inflammatory infiltrate.

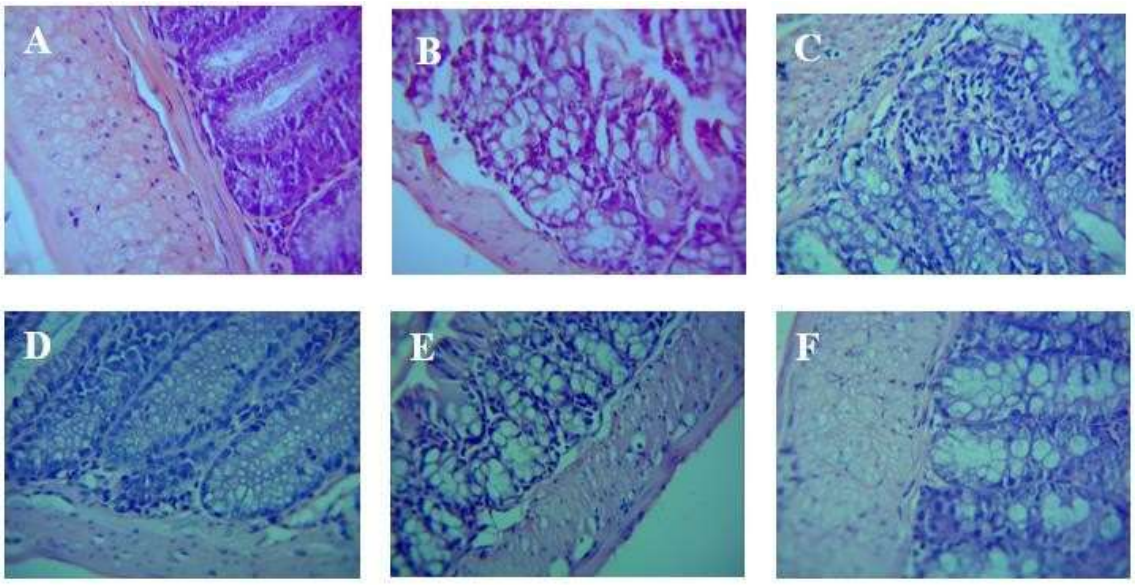

Figure 17: Haematoxylin and Eosin stained coronal sections of colon 40X magnification.

(A) Control (B) Diseased Control (C) Positive Control (D) Treatment with Chrysin (E) Treatment with Probiotics (F) Treatment with Chrysin and Probiotics

CHAPTER 5

5.0 Discussion

Depression is an exceptionally pervasive mental health condition that affects people from all walks of life. Characterized by persistent feelings of sadness, hopelessness and difficulty finding joy in activities previously enjoyed, it can have a significant impact on emotional and physical wellbeing as well as daily function. The indicators associated with depression are diverse - they may include but are not limited to constant despair or hollowness inside, lack of interest in past pleasures or hobbies, changes in appetite/weight imbalance due to overeating/under eating; excessive sleeping or insomnia; exhaustion coupled with low energy levels; feeling worthless till the extent where guilt arises without any legitimate cause behind ;concentration which involves making decisions seems too laborious for them alongwith the thought concerning suicide . Depression can also have physical effects on the body, such as headaches, digestive problems, and chronic pain. Additionally, it can contribute to social isolation and difficulties in personal relationships, as well as difficulties at work or school. Depression can lead to self-harm or suicide in extreme instances. TCAs and MAO inhibitors are available as treatments for depression, but they have numerous undesirable side effects, including sedation, hypotension, impaired vision, and parched saliva. Due to their effects on the cardiovascular system, an overdose of TCAs may also be fatal and life-threatening. Thus, depression requires supplementary or complementary therapy options.

In this study, previously isolated *Limosilactobacillus fermentum* strains 7b, 19b, 18 were selected based on their antioxidant and antidepressant potential. Its safety and survivability in GIT have already been evaluated by Muneera (2016). In 2018, Hamza and Noor assessed the antidepressant properties of these probiotic strains through in-vivo trials. As there is disruption of neurotransmitters in depression,

Limosilactobacillus fermentum 7b, 19b, 18 can help maintain the normal level of neurotransmitters by preserving the microbiota in the intestine. Depression has been treated using *Limosilactobacillus fermentum* and other probiotics. In addition, the antidepressant effect is enhanced by the addition of Chrysin, a polyphenol, in a symbiotic formulation. Chrysin has antidepressant properties and has been shown to increase serotonin and other neurotransmitters such as GABA, dopamine, etc.

The present study showed that supplementation of *Limosilactobacillus fermentum* 7b, 19b, 18 at a dosage of 1x109 CFU per day and Chrysin (25mg/kg) on mice CUMS model has a neuroprotective effect against depression due to their antioxidative and antidepressant properties. The experiment was carried out for 28 days. Chronic unpredictable mild stress was given to all groups for 28 days except the negative control group. TP, TPC were given probiotics and chrysin + probiotics whereas TC was given chrysin only along with CUMS. PC, Positive control group was given fluoxetine (18mg/kg) along with CUMS. Anxiety, depression and memory impairment was detected through various behavior tests. The results showed that the supplementation of symbiotic substantially attenuated the depression induced by CUMS.

Anxiety levels were measured by elevated plus maize test. The number of open arm entries in EPM suggested that the diseased group exhibited anxiety-like effects, as compared to the number of entries made by the control group. Similarly, the amount of time spent in the open arm was significantly less than the control group, demonstrating anxious behavior. Oral administration of probiotics ameliorates anxiety-like behavior in both animal and human studies. From the 18th day of gestation until birth, pregnant C57BL/6 female rodents were orally gavaged with *Limosilactobacillus rhamnosus* LGG®, and their progeny were also gavaged for 5 days. LGG® colonization enhanced the expression of GABA receptors, BDNF, and serotonin transporter, resulting in anti-anxiety-like behavior in adulthood (Zhou et al., 2021). *Limosilactobacillus rhamnosus* (JB-1) was administered to male C57BL/6 rodents for 28 days, followed by protracted social defeat. Compared to the placebo group, probiotic-treated mice

responded to social tension with less anxiety. JB-1 colonization led to an increase in IL-10 regulatory T-cells and a decrease in dendritic cell activation. (Bharwani et al., 2017). In a study, chrysin reverses depressive behavior, astroglial metabolic activity, and impaired neuronal activity in depression model of CUMS in C57BL6 mice (Mishra et al., 2020). This study confirmed the evidence that probiotics are beneficial for reducing anxiety-like behavior. In comparison to the disease group, the combination of *Limosilactobacillus fermentum* consortium and chrysin significantly reduced anxiety-like behavior in the control, TPC, TP, and TC groups. An increase in entries and time spent in open arm suggests a reduction in anxious behavior. Treatment with TPC is effective in reducing anxiety-like behaviors associated with depression.

An open-field test was carried out to determine the exploratory behaviors and anxiety levels. *Limosilactobacillus fermentum* oral administration improves anxiety like behavior in mice in open field test by regulating by regulating monoamine, serotonin and dopamine levels and BDNF pathways (M. Gao et al., 2023). For 28 days, *Limosilactobacillus fermentum* was administered to male C57BL/6 mice, which were then subjected to chronic, unpredictable, mild stress. Mice treated with probiotics had a decreased anxiety-like response to chronic stress as compared to placebo group (Chudzik et al., 2021). In female mice, chrysin increased the levels of BDNF, NGF, and Na+,K+-ATPase, all of which decreased in response to chronic stress, demonstrating its antidepressant effects (Filho et al., 2015a). In current study, groups treated with probiotics and chrysin (25mg/kg) displayed more exploratory activity by spending more time in central region like control group. Similarly, probiotics and chrysin (25mg/kg) showed better rearing behavior than disease control and positive control group. Thus it can be proposed that combination of probiotics and chrysin is very effective in reducing anxiety like symptoms in depression.

The Forced Swim Test is commonly used to determine an animal's level of depression. During this test, the duration an animal spends immobile indicates its depressive state - those with greater levels of

depression immobilizing for a longer period than those with less. The constant struggle against drowning during testing suggests that animals are still attempting to stay afloat and escape their situation, whereas depressed individuals show evidence of hopelessness by giving up earlier on this effort (Takeda et al., 2002). In a single arm study, adult mice after administration of the living probiotic bacterium *Limosilactobacillus fermentum* strain JDFM216 modulates immune response with gut microbiota and improves cognitive behavior (Park et al., 2020). In another investigation, chrysin was shown to ameliorate the depressive-like behavior and hippocampal dysfunction caused by OB in rodents (Filho, Jesse, Donato, Del Fabbro, Gomes de Gomes, et al., 2016b). In the present study, probiotics and chrysin exhibited normal behavior, similar to that of the control group, whereas the diseased group displayed relatively more immobility than the other groups, indicating relatively higher levels of depression. Hence, we can assume that combination of probiotics and depression is helpful in treating depression like behavior of animals.

In rodents, sucrose preference test is a measure to access depression. It is the most frequently used method for determining the reward sensitivity of rodents with the consumption or preference for delectable sweet solutions, such as sucrose or saccharin. In order to determine the antidepressant effect of probiotics in rodents, a study was conducted on the novel probiotics *Bifidobacterium longum* and *L. rhamnosus* against chronic unpredictable moderate stress. According to the findings these particular strains performed better than the diseased group did in terms of depression. This is due to the fact that *Bifidobacterium longum* and *L. rhamnosus* balanced the 5-HT concentrations in the frontal cortex and hippocampus (Li et al., 2019b). Due to their antidepressant properties, flavanols such as chrysin are believed to have therapeutic potential in the treatment of depression and other neurodegenerative disorders. In order to examine the effects of this flavanol on the behavioral reactions of mice, chrysin was administrated to mice subjected to CUMS. It was shown that mice given chrysin up-regulated BDNF and NGF levels as chrysin act as antidepressant because mice in this group prefer sucrose over plain water (Filho et al., 2015b).

This study provides further support for the existing evidence suggesting that probiotics and chrysin are beneficial in reducing the symptoms of depression. Combination therapy of *Limosilactobacillus fermentum* (7b, 18, 19b) and chrysin caused significant decrease in depression in TPC group as compared to probiotic and chrysin given individually. Antidepressant behavior was measured by the preference of drinking sucrose over plain water. So, it is found that a combination of both can show more promising results in overcoming depression.

Limosilactobacillus fermentum PS150 treatment of depression induced by CMS led improved spatial memory. This improvement was assessed by MWM. The improvement was rendered by a decrease in inflammation and increase in serotonin levels in probiotic treated group (Y.-W. Liu et al., 2019). The administration of *Bifidobacterium Lactis* Probio-M8 to APP/PS-1 transgenic mice increased α and β-diversity of their gastrointestinal tracts. Treatment with probiotics ameliorated cognitive decline in APP/PS-1 transgenic rodents, as measured by the percentage of spontaneous alternations and the percentage of contacts with the novel object (Yun et al., 2020). Crlj: CD-1 (ICR) female mice were subjected to chronic unpredictable mild stress and separation, which induced depression. In rodents with depressive-like behavior, chrysin treatment reduced depression via the NF-B signaling pathway that accelerated hippocampal microglia activation and cognitive impairment (Tang et al., 2019).

In this study, the disease group demonstrated a significant decline in cognition and performed poorly on all memory assessments. Both spatial and reference memory parameters exhibited a significant decline. As indicated by escape latency, both long-term spatial memory and learning were substandard. The diseased group spent less time in the target quadrant during the MWM than the other groups. Similarly, the number of platform crossings was lowest in the diseased group. In contrast, probiotics and chrysin demonstrated a significant improvement in all memory test parameters. The cognitive enhancement was comparable to that of the control group. This combination of probiotics is effective in reversing the cognitive decline associated with depression. Our study further included histological

evaluation of the brain hippocampus in mice as well as the colon. The results from the histological examination further supported our findings. In the H&E stained mice brain hippocampus, we observed improved neuron structures and reduced pyknotic nuclei in the groups treated with probiotics and chrysin compared to the diseased group. Chronic Unpredictable Mild Stress (CUMS) can lead to neurodegeneration due to increased inflammation and reactive gliosis (Y.-W. Liu et al., 2019). The cell counts achieved through treatment with probiotics, chrysin, and fluoxetine were comparable to those in the negative control group. Additionally, the H&E stained mice colon showed improved healthy epithelial tissues and villi structures compared to the diseased group. CUMS caused inflammatory cell infiltration, villus atrophy, and epithelial crypt loss (Y.-W. Liu et al., 2019) (Li et al., 2019b). Fluoxetine demonstrated efficacy in treating depression and aiding in the recovery from brain injuries caused by trauma. Similarly, *Limosilactobacillus fermentum* and chrysin improve BDNF and NGF levels and neurotransmitters, serotonin and dopamine thus alleviating depression (Filho et al., 2015a). Hence, the administration of *Limosilactobacillus fermentum* and chrysin as a pretreatment option demonstrated positive effects on maintaining the morphology of neurons in the hippocampus and epithelial tissues in the colon of the TPC group compared to the group exposed to CUMS.

CHAPTER 6

6.0 CONCLUSION AND FUTURE PROSPECTS

The present study proved that combination therapy of probiotics and chrysin was effective in improving depression and anxiety-like behavior in mouse model of CUMS. Since, probiotics and chrysin 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 and chrysin were so effective in treatment of depression. Future prospects include genomic analysis through RT-PCR and 16s RNA profiling of fecal microbial diversity to determine the effect of probiotic and chrysin oral consumption on α-diversity of microbes in gut. Furthermore, effect of probiotic and chrysin consumption must also be studied on neurotransmitters modulation to give further insight as to how gut microbiota attenuates the severity of disease symptoms.

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1. Research Project Title: Therapeutic effect of indigenous probiotic Lactobacillus spp. Strains along with functional food in ameliorating chronic unpredictable mild stress in mice.

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-01/01

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
Dept. of Plant Biotechnology
ASAB, NUST Islamabad

Signature of the Head / Deputy Head of Thematic Research

Pro-Rector (RIC)
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The therapeutic effect of *Limosilactobacillus fermentum* strains and chrysin in chronic unpredictable mild stress mice model

By

Iqra Mutiullah

NUST[illegible]

Master of Science in Industrial Biotechnology

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