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PROBIOTIC ADMINISTRATION AS AN ADJUVANT THERAPEUTIC TREATMENT FOR ANXIETY, DEPRESSION, AND COGNITIVE IMPAIRMENT AMONG HYPOTHYROID PATIENTS

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by

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DEDICATION

My ultimate goal in writing this thesis was to become further educated on the topic of mental health in order to better understand myself and those around me.

I dedicate this work to those suffering from mental illnesses of all variations,

And specifically, to my loved ones who struggle daily with their mental health.

Individuals must realize the reality of mental illness as just that—an illness.

Depression is not a choice.

Anxiety is not a choice.

Mental illness is not a choice.

ACKNOWLEDGMENTS

I would say that there are too many people for me to thank, but I don't believe there is such thing as having too many amazing and inspiring people in one's life.

I would first like to thank my family for being the biggest support group throughout my life.

Thank you, Mom and Dad, for being such wonderful parents and for teaching me discipline, respect, independence, the value of hard work, and kindness—things I will always have to my advantage, thanks to you.

Thanks siblings (Jess, Alyssa, and Michael) for being my forever-best-friends, even if not by choice. I give you all the credit for my competitive drive. I guess that's what years of losing at Monopoly does to a person.

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Abstract

Hypothyroidism is a form of thyroid dysfunction that occurs when the thyroid gland does not make and secrete enough thyroid hormones to regulate certain processes in the body. Because thyroid hormones take part in many bodily functions, hypothyroidism can cause a large range of symptoms. Current research indicates that some strains of probiotics have beneficial effects on certain neurological and inflammatory diseases, leading to the impression that they can be used therapeutically for effective treatment of different mental health issues such as anxiety, stress, depression, and impaired memory. Because hypothyroidism often leads to such mental symptoms, it may be possible that probiotic treatments reverse these symptoms in hypothyroid patients. The current proposal aims to 1) observe the effects of probiotic strains *Bifidobacterium longum*, *Bifidobacterium breve*, and *Bifidobacterium infantis* on hypothyroid patients suffering from minor to severe anxiety and depression and 2) observe the effects of the same strains on cognition in hypothyroid male Sprague-Dawley rats. It is predicted that probiotics will reduce depressive and anxiety symptoms in humans and improve memory and learning in rats.

Keywords: probiotics, hypothyroidism, *Bifidobacteria*, depression, anxiety, cognitive impairment, learning and memory, inflammation

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LIST OF ABBREVIATIONS

ASD	Autism spectrum disorder
ANS	Autonomic nervous system
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
DIT	Diiodotyrosine
ENS	Enteric nervous system
ELISA	Enzyme-linked immunosorbent assay
EPSP	Excitatory postsynaptic potential
FST	Forced swim test
GABA	Gamma-aminobutyric acid
GI	Gastrointestinal
GAD	Generalized anxiety disorder
GMB	Gut-microbiome-brain
hsCRP	high-sensitivity C-reactive protein
HPT	Hypothalamus-pituitary-thyroid axis
IBS	Irritable bowel syndrome
LT_4	Levothyroxine

LTD	Long-term depression
LTP	Long-term potentiation
MDD	Major Depressive Disorder
MS	Maternal separation model
MIT	Monoiodotyrosine
NMDA	N-methyl-D-aspartate glutamate receptor
PD	Panic disorder
PNS	Peripheral nervous system
PSD	Postsynaptic density
PTU	Propylthiouracil
SCFA	Short chain fatty acid
TG	Thyroglobulin
TH	Thyroid hormone
TSH	Thyrotropin/Thyroid- stimulating hormone
TRH	Thyrotropin-releasing hormone
T_4	Thyroxine
T_3	Triiodothyronine
TrkB	Tyrosine kinase receptor

INTRODUCTION

Hypothyroidism is a disorder characterized by the low production of thyroid hormones by the thyroid gland, a structure in the body essential for regulation and use of energy (*Figure 1*).[1,2] Thus, hypothyroidism is associated with slower metabolic functions and may contribute to poorer quality of life. Five out of every one hundred people in the United States are impacted by hypothyroidism, with the majority of those affected being women.[3] Hypothyroidism has several different causes, with the most common cause being autoimmune thyroid disease.[2] The thyroid gland and thyroid hormones affect many different biological processes, leading to a wide range of symptoms when dysfunction occurs. Commonly associated with hypothyroidism are symptoms of depression, anxiety, and neurocognitive impairment, which may not be reversed with thyroid hormone replacement therapy.[4] Notably, psychiatric symptoms often present earliest and most conspicuously in hypothyroid patients.[5] Consequently, hypothyroidism may first be misdiagnosed as psychiatric disturbance.[5]

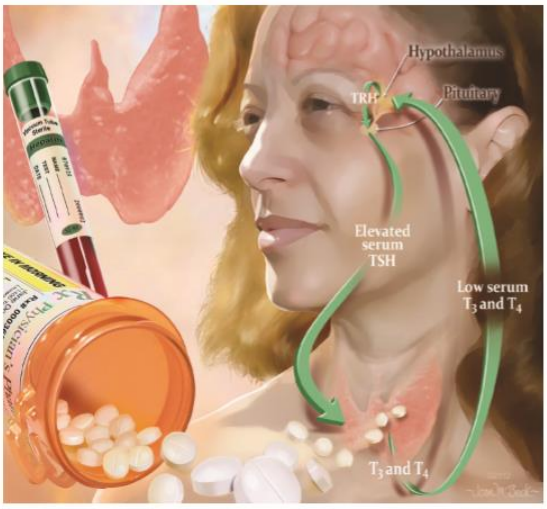


Figure 1. Hypothyroidism is characterized by low serum T_3 and T_4 thyroid hormone, which may lead to elevated serum levels of thyroid stimulating hormone (TSH).[2]

Patients with hypothyroidism may go years without proper diagnosis or treatment. Since symptoms are so nonspecific, and may be attributed to other health issues, it can be difficult to identify hypothyroidism as the primary cause. Approximately 13 million people in the United States are currently living with undiagnosed hypothyroidism.[2] Misdiagnosis and persistence of

symptoms despite treatment attempts contribute greatly to the frustration of being unable to improve quality of living. Patients suffering from undiagnosed hypothyroidism may experience fatigue, weight gain, or chronic pain, with no certainty of the cause. Proper diagnosis of hypothyroidism is a crucial first step in patients attaining a higher quality of life. The next crucial step is to determine the most effective way to treat symptoms that still persist even after normal thyroid hormone levels are reached. Some symptoms such as weight gain and fatigue have been reported to remain despite treatment.[2] However, the treatment of persistent symptoms of anxiety, depression, and cognitive impairment will be the focus of the following discussion.

HYPOTHYROIDISM

The Thyroid and its Functions

The thyroid gland is a hormone secreting structure in the endocrine system, most easily identified by its butterfly-like shape (*Figure 2*).[6] Two ovaloid lobes lie on either side of the trachea, joined by an isthmus which crosses the trachea ventrally.[7] Groups of spherical follicles filled with fluid make up the lobes of the gland.[7] Follicles are formed by a single layer of follicular epithelial cells, also known as thyrocytes, which contain tight junctions that form a strong barrier and prevent diffusion of transmembrane proteins through the apical and basolateral sections of the plasma membrane.[8]

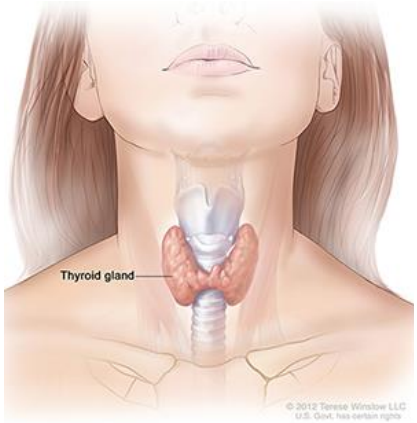
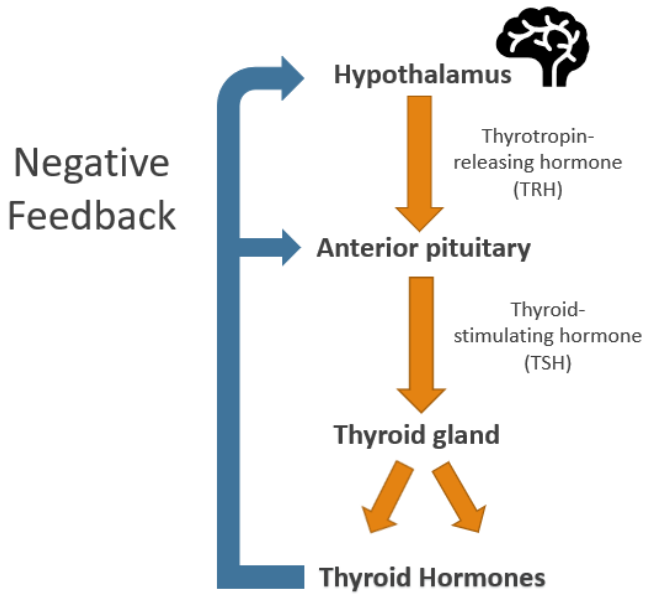


Figure 2. Thyroid gland.[3]

Follicular cells along the walls of the thyroid gland use iodine to produce a glycoprotein called thyroglobulin (TG), which is necessary for the production of thyroid hormone (TH).[6] Absorption of iodide occurs in the gastrointestinal tract, where it travels through the bloodstream to the basolateral plasma membranes of follicular cells.[8] Iodide is converted to iodine by oxidation in the follicular lumen, where it then binds to the tyrosine residues.[8] Iodine atoms within the body attach to tyrosyl residues of TG, producing colloid, which is stored in the central cavities of follicles and used to produce thyroid hormone.[6] Monoiodotyrosine (MIT) and diiodotyrosine (DIT) are produced, containing one or two iodines respectively.[6] MIT and DIT are then coupled to form the two types of thyroid hormone, which are both made up of iodine atoms and two tyrosine amino acids.[6] Thyroxine (T_4) contains four iodine atoms, while triiodothyronine (T_3) contains three. TH is produced through receptor binding of thyrotropin, or

thyroid- stimulating hormone (TSH), onto follicular cells and may be produced in large amounts outside of the cell.[6] T_4 is the major hormone produced by the thyroid gland. However, T_3 is more active in the body.[9] T_3 has a major role in signaling the negative feedback loop of the hypothalamus-pituitary-thyroid (HPT) axis (*Figure 3*). The HPT axis regulates the concentration of T_3 and T_4 in the body. Thyrotropin-releasing hormone (TRH) in the hypothalamus region of the brain has important roles in energy metabolism, feeding behavior, heat production, and regulation of certain autonomic nervous system processes.[9] In addition, release of TRH from the hypothalamus stimulates TSH synthesis and release. TSH is an important hormone in the pituitary gland that regulates iodine uptake by thyroid cells, contributes to thyroid cell differentiation and growth, and stimulates TH production and release by the thyroid gland.[9] Overall, concentration levels of T_3 in the body negatively regulate how much or how frequently TRH is released from the hypothalamus, and therefore how much TSH is released from the pituitary gland .[9] When TH levels are high, a signal is sent to the hypothalamus to reduce release of TRH, which results in lower production of hormones. Likewise, if TH levels are too low, TRH release will increase in order for more hormones to be synthesized by the thyroid.[3,9] Although less is known about the mechanism involved, THs may also directly inhibit release of TSH from vesicles in the pituitary

Figure 3. The HPT axis uses negative feedback to regulate hormone release and synthesis.



gland, which also results in lower production of hormones.[9]

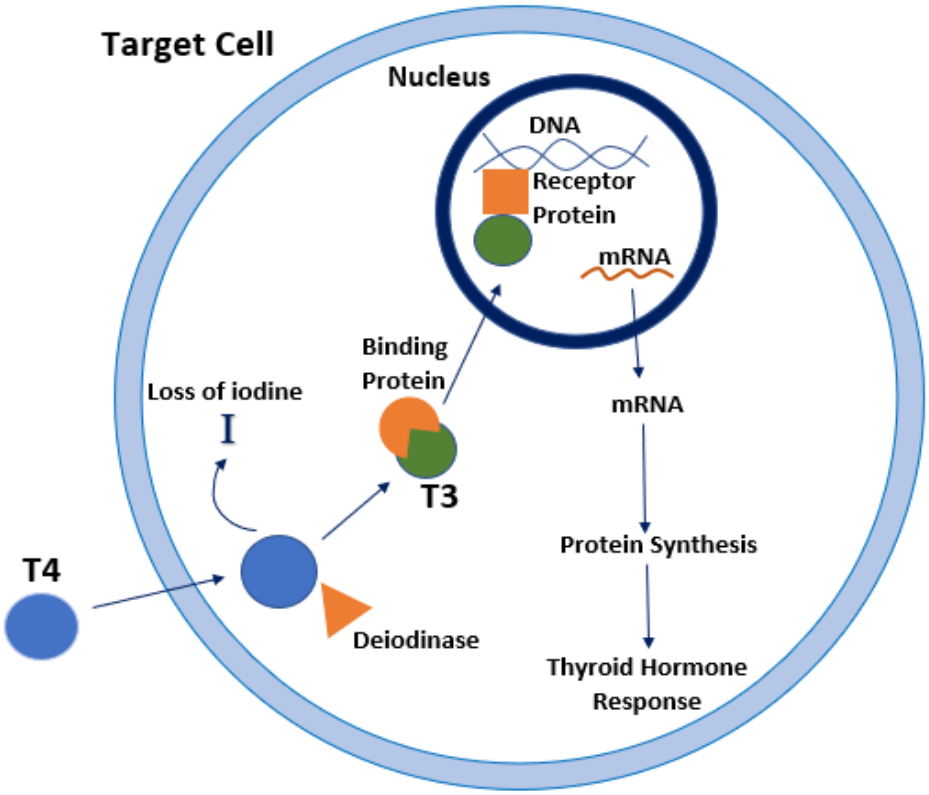
Both T_3 and T_4 are made and secreted by the thyroid, but 80% of serum T_3 is converted from T_4 through the removal of an iodine by a deiodinase. There are three types of deiodinases (D1-D3), which differ in preferred substrates, tissue location, physiological function, and response to excess T_4 (*Table 1*).[9] Importantly, D1 is the only deiodinase inhibited by administration of propylthiouracil (PTU), a compound used to induce hypothyroidism in animal models, or to treat hyperthyroidism. Inhibition of D1 causes decreased conversion of T_4 to T_3, which decreases the amount of serum T_3 by 25% and limits the biological effects of TH.[9,10]

Table 1. Deiodinases (D1-D3) differ in which substrates they bind to, which tissues they are located, physiological function, and how they respond to excess amounts of T_4.[10]

	Dl	D2	D3
AA residue at catalytic site	Selenocysteine	Selenocysteine	Selenocysteine
	Outer (5′) and inner (5) ring deiodination	Outer (5′) ring deiodination	Inner (5) ring deiodination
Preferred substrate	5′: rT3>T4>T3 5: T4S>T3S	T4>rT3>T3	T3>T4
Inhibition by PTU	+	–	–
Tissue location	Liver, kidney, thyroid, pituitary	CNS, pituitary, BAT	Skin, CNS, Placenta
Subcelullar location	Plasma membrane	Endoplasmatic reticulum	Plasma membrane
Physiological role	Production of plasma T3	Production of intracellular and plasmaticT3	T3 degradation
Response to excess of T4 (except thyroid)	Increase	Decreases	Increase

The vast majority of THs bind to blood plasma proteins once released by the thyroid.[9] Upon entering a cell's nucleus, TH binds to receptors, which then initiate transcription of mRNA and lead to protein synthesis (*Figure 4*).[6] Since TH reacts with most cells within the body, the thyroid gland affects numerous biological processes.[6] Proteins synthesized through this process affect the nervous, cardiovascular, muscular, skeletal, gastrointestinal (GI), reproductive, and integumentary systems, as well as basal metabolic rate and macromolecule (carbohydrate, lipid, protein) metabolism.[6]

Figure 4. T_4 enters the cell where it is converted to T_3. It then binds to receptors, which initiate transcription of mRNA and lead to protein synthesis.



Normal TH levels are essential for proper brain development and function. THs play a role in myelination of axons, cell migration, and differentiation of neurons. They also regulate the expression of several genes involved in synaptic plasticity and memory, such as CAMKII and neuroganin.[9] It has been shown that inadequate TH levels leads to a decrease in neurogenesis of the hippocampus, a brain region highly involved in cognition, and more specifically in learning and memory.[11] Effects of insufficient TH levels are much more severe when present during child development.[11] The severity of these effects emphasizes the importance of THs for normal brain development and function.

THs are essential for normal development of the retina, tectorial membrane, hair cells, and middle ear ossicles. Furthermore, T_3 is necessary for the processing of sound information. Thus, altered TH levels, specifically during development, may prevent proper sensory functions.[9] THs also affect heart development and function. T_3 controls gene expression of several genes involved in cardiomyocyte, heart cell, growth and function. High T_3 levels may lead to excessive stimulus of the heart, causing cardiac hypertrophy, which is enlargement of the heart. Low T_3 levels may lead to slowed heart rate, known as bradycardia, which can cause dizziness and weakness.[9] Furthermore, THs play important roles in bone development, linear growth, and maturation, as well as regulation of bone reabsorption which promotes skeletal strength.[9]

Finally, THs are essential for the regulation of energy and nutrient metabolism. They control energy utilization and thermogenesis.[9] Body temperature is maintained through obligatory and facultative methods, which are both stimulated by THs. Obligatory thermogenesis involves the constant maintenance of standard body temperature (37°C), while facultative thermogenesis involves the induction of shivering reflexes when the body is exposed to cold temperatures.[9] Low TH production causes cold intolerance and may lead to development of

hypothermia in extreme cases, while high TH production causes heat intolerance.[9] THs regulate the degradation and synthesis of lipids, and high levels of TH increase lipolysis. In addition, THs increase synthesis and oxidation of fatty acids in the liver, as well as their conversion to triglycerides, and stimulate synthesis of cholesterol and its uptake. Low TH levels lead to reduced metabolic function, while high levels lead to excessive metabolism. THs regulate protein synthesis and degradation, with high TH levels leading to increased degradation and loss of protein in muscles. THs also effect metabolism of carbohydrates and have recently been found to stimulate appetite. Evidently, the thyroid is crucial for normal development and metabolic function. Consequently, its dysfunction can lead to malfunctions within any of the mentioned systems and processes.

Thyroid Dysfunction: Hyper- and Hypothyroidism

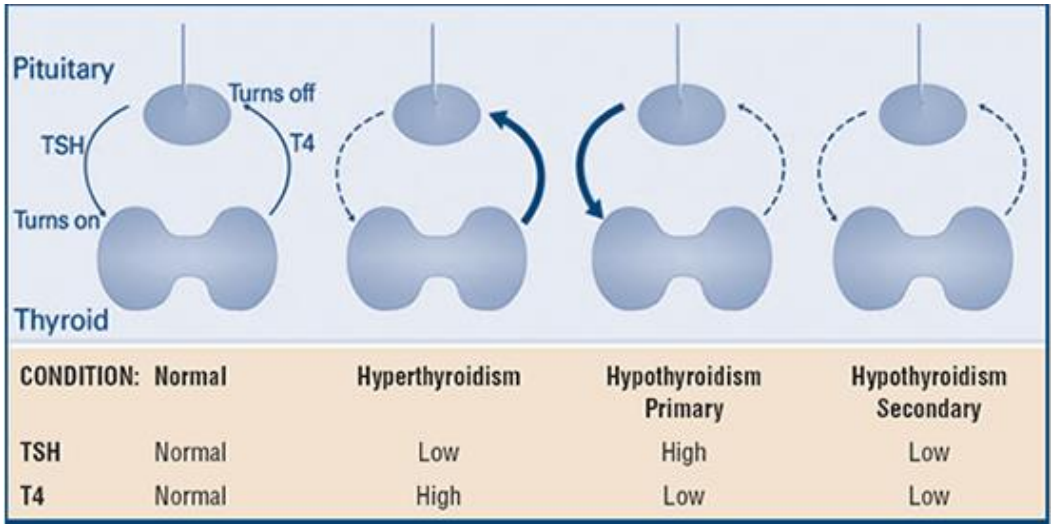
Thyroid dysfunction may arise in several ways (*Figure 5*). One such way is hyperthyroidism, a condition characterized by the high production and secretion of thyroid hormones.[12] The most common cause of hyperthyroidism is an autoimmune disorder called Grave's disease. This disease involves the production of antibodies which bind to TSH receptors and stimulate the synthesis of THs. Thus, Grave's disease is characterized by low levels of serum TSH due to negative feedback by high amounts of serum TH.[12,13]

Hyperthyroidism has a prevalence of 1.2% in the United States.[13] Symptoms are broad and nonspecific, making the disease difficult to diagnose. However, testing serum TSH levels is usually an effective way to correctly diagnose the disease[13] Patients with hyperthyroidism may have increased heat production and intolerance to heat, low serum cholesterol levels, protein loss in muscles and various tissues (leading to weight loss), increased appetite, decreased bone integrity and linear growth, and increased heart rate, which may lead to heart failure.[9,14]

Hyperthyroid patients may also present neurological symptoms such as anxiety, rapid speech, sleep disturbance, and psychosis.[13] Persistence of both physical and mental symptoms can be detrimental to the health of these patients.

There are few treatment options for hyperthyroidism. Antithyroid medications that inhibit TH production, such as methimazole or PTU, may be used as initial treatment for hyperthyroidism. However, the risk of adverse effects may increase with their long-term use.[13] Another treatment option is ablation of the thyroid gland through uptake of radioactive iodine. Once the thyroid gland is "killed", hypothyroidism will most likely develop after several months. The final treatment option involves surgical removal of the thyroid, known as thyroidectomy. Similar to radioactive iodine ablation, hypothyroidism will generally develop after several months.[13] Fortunately, hypothyroidism is safer and easier to treat long-term.[15]

Figure 5. Hyperthyroidism is characterized by low TSH and high TH. Primary hypothyroidism is characterized by high TSH due to lack of TH.[87]

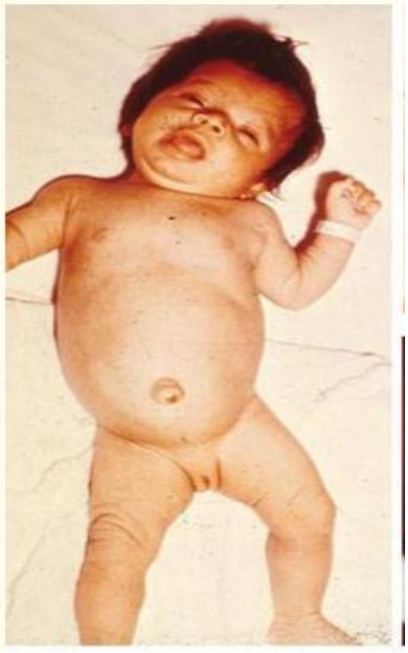


Hypothyroidism is a form of thyroid dysfunction involving low production and secretion of thyroid hormones. Normal thyroid function involves interaction with the hypothalamus, a region of the brain.[3] The hypothalamus determines when TH levels are too low or high. In response to TH imbalance, a signal is sent to the pituitary gland, which sends TSH to the receptors on follicular cells involved in TH synthesis and release.[3] In hypothyroidism, the thyroid gland is lacking in its ability to respond to TSH. Thus, thyroid hormone is not produced efficiently in underactive thyroids.[3] Because the thyroid gland plays a large role in regulating the body's use of energy, many biological processes slow down with lack of thyroid hormone.[3]

There are several causes of hypothyroidism, with the most common cause being an inflammatory disorder called Hashimoto's disease.[3] Like Grave's disease, Hashimoto's is an autoimmune disorder that depends on both genetic and environmental factors.[16] The disease leads to the gradual atrophy, or wasting away, of the thyroid gland after being invaded by lymphocytic cells of the immune system. Other than the fact that lymphocytes are involved, the pathogenesis of Hashimoto's is not fully understood.[16]

Congenital hypothyroidism is a serious disorder that occurs in infants born with a malfunctioning or underdeveloped thyroid. Symptoms include low energy, increased sleep, difficulty with feeding, constipation, and jaundice. Physical deformities may be present as well (*Figure 6*).[17] Congenital hypothyroidism may be permanent, demanding treatment throughout life, or transient, waning after several months. Risk of permanent congenital hypothyroidism is more likely to occur in iodine-

Figure 6. An infant displaying congenital hypothyroidism.[17]



deficient countries.[17] Permanent congenital hypothyroidism is frequently accompanied by mental retardation, impaired cognition, and sometimes hearing loss.[9]

One cause of a temporary form of hypothyroidism is thyroiditis. Thyroiditis is inflammation of the thyroid that first causes hyperthyroidism by leaking thyroid hormones. After several months, the thyroid may enter into hypothyroidism before returning to normal.[3] As mentioned before, treatment options for hyperthyroidism generally lead to the development of hypothyroidism. Thus, hypothyroidism may occur through whole or partial removal of the thyroid gland when needed to treat hyperthyroidism or thyroid cancer.

Like hyperthyroidism, patients with hypothyroidism have a wide range of symptoms including poor mental health, cognitive impairment, sexual dysfunction, decreased macromolecule metabolism, lowered basal metabolic rate, weight gain, joint pain, slowed reflexes and muscle actions, and irregular heart rates.[4,6,18,19] Persistence of symptoms may contribute to more serious health issues such as hypertension, abnormal lipid levels, infertility, or neuromuscular dysfunction.[2]

Mental health issues such as anxiety, stress, and depression are some of the most common yet most overlooked health issues in the medical realm. Depression alone may cause cognitive impairments, emotional imbalance, memory deficiencies, lack of motivation, limited motor function, and dissociation from society.[20] Depressive symptoms often precede hypothyroidism, and in other cases hypothyroidism may increase the likeliness for depressive disorders to develop.[21] Feelings of sadness, hopelessness, worthlessness, emptiness, anxiety, and restlessness are characteristic of depressive disorders. People suffering from depression often lose interest in activities previously enjoyed, avoid social interaction, have abnormal eating and sleeping habits, have trouble concentrating, and in some cases, experience suicidal thoughts or

attempt suicide.[22] There are also generally other chronic illnesses associated with depression, such as irritable bowel syndrome (IBS). The costs of therapy, antidepressants, reduced motivation in the workplace, and additional treatment of secondary illnesses make depression a very large financial medical burden.[20]

Hypothyroid patients may also present symptoms that are also characteristic of generalized anxiety disorders (GADs), such as anxiety, sleep difficulty, fatigue, and attention deficits.[21] Additionally, hypothyroid patients may display symptoms that also occur in patients with panic disorders (PDs) during panic attacks, such as heart palpitations, irregular breathing, and increased sweating.[21] Similar to depressive disorders, anxiety disorders often precede the development of thyroid dysfunction. However, patients with thyroid dysfunction are more likely to develop anxiety disorders.[21] Overall, symptoms of anxiety and depressive disorders may overlap with those of hypothyroidism, making it difficult to determine which is contributing to the presence of those symptoms and what type of treatment should be used.

Hypothyroidism also impairs synaptic plasticity and cognition, specifically learning and memory.[11,23] Synaptic plasticity is the ability for neuronal synapses to strengthen or weaken in order to mediate efficient storage of information in the hippocampus, both short-term and long-term.[24] The two types of long-term synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD). LTP is the strengthening of synapses, while LTD is the weakening.[24] Increased LTP is associated with enhanced learning and memory, while increased LTD (or decreased LTP) is associated with impaired learning and memory processes.[23] Hypothyroidism causes deficits in LTP, which is likely a contributor to impairment of learning and memory in patients.[23]

Symptoms vary greatly between hypothyroid patients, and some may be subtle or not present at all.[2] Once diagnosed, the primary treatment for hypothyroidism is thyroid hormone replacement therapy through administration of levothyroxine (LT_4), a synthetic form of T_4.[25] This synthetic form of T_4 is then converted to serum T_3. Thus, levothyroxine treatment alleviates symptoms of hypothyroid patients by normalizing serum T_4 and T_3, and consequently TSH.[25,26] However, it has been reported that attaining normal thyroid hormone levels, called euthyroidism, does not guarantee a reversal of all symptoms associated with hypothyroidism.[25] While antidepressant and anxiolytic treatments are generally effective for depressive and anxiety symptoms, long-term use of these medications may increase the risk of unwanted effects and health complications in patients.[27,28] Neurocognitive impairments, specifically in memory and attention, have also been reported to persist in hypothyroid patients despite adequate, long-term levothyroxine treatment.[26] Interestingly, symptoms of depression, anxiety, and neurocognitive impairments correspond with ailments that have been demonstrated to be helped by probiotic administration.[29–34]

PROBIOTICS

The Gut-Brain Axis

It has long been understood that brain function is connected to the microbiome in the GI tract. The GI tract is extremely complex, spanning five meters, containing the majority of immune cells within the body, as well as hundreds of millions of neurons. More so, the microbiome of the GI tract contains trillions of cells and hundreds of varying species.[35] The brain-gut axis is a bidirectional communication system that involves the central nervous system (CNS) and enteric nervous system (ENS).[36] The ENS is a branch of the peripheral nervous system (PNS), as is the autonomic nervous system (ANS), which is responsible for involuntary bodily responses.[35] The ANS may be further divided into sympathetic and parasympathetic. Nerves within these systems may send signals and synapse directly onto the GI tract. The vagus nerve, an important parasympathetic nerve, connects the hindbrain, esophagus, and GI tract.[35] Stimulation of the vagus nerve can produce anti-inflammatory responses, providing evidence of its role in gut-brain communication.[37] The CNS encompasses the spinal cord and brain, and the ENS encompasses the gastrointestinal system, regulating its functions through neuronal receptors.[35] Connections between microbiota of the GI tract and the brain have been shown to have roles in gastrointestinal disorders, obesity, anxiety, stress, depression, cognition, and possibly even autism.[36,38,39]

Importance of the Gut Microbiome

Gut microbiota have a mutualistic relationship with their hosts. Their presence is imperative for immune responses, fat distribution, absorption of nutrients, and gut contractions, and gut integrity.[40] Alterations of the gut microbiome early on in life may have detrimental effects on health. For example, when infants are delivered vaginally, they acquire microbes from

the existing microbiome of the mother. However, when delivered by cesarean section, infants may not acquire a wide variety of microbial populations.[41] Prenatal stress may also influence the population of microbes in an infant's microbiome. An altered gut microbiome at birth has been shown to increase risk of infants manifesting an autoimmune disease later in life.[41] Antibiotic use early in life may also alter the microbiome, impacting development, metabolism, immune health, and behavior.[41] Germ-free rodent models with sterilized gut microbiomes are frequently used for microbiome studies. These models tend to display significant impairments in behavior, cognition, and neurochemistry (*Figure 7*).[39] Fecal transplant studies are done to observe the effects of different microbial populations on behavior and physiology. Infection studies are also done to observe the effects of pathogens on behavior and physiology (*Figure 8*).[40]

Figure 7. Phenotypes exhibited by germ-free mice. Decreased memory, sociability, anxiety, and BDNF. Increased locomotion, self-grooming, serotonin, adrenocorticotropic hormone, corticosterone.[39]

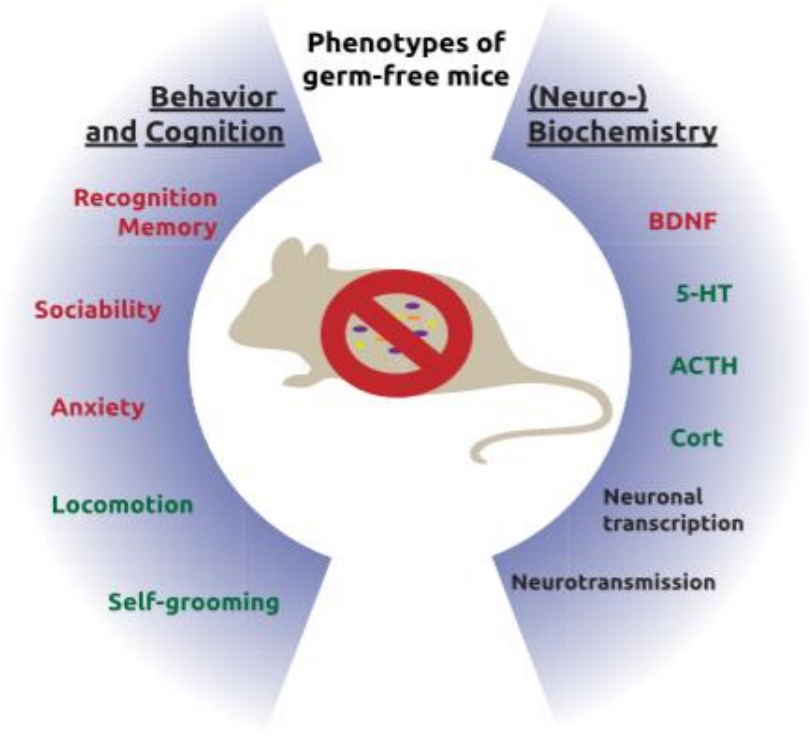
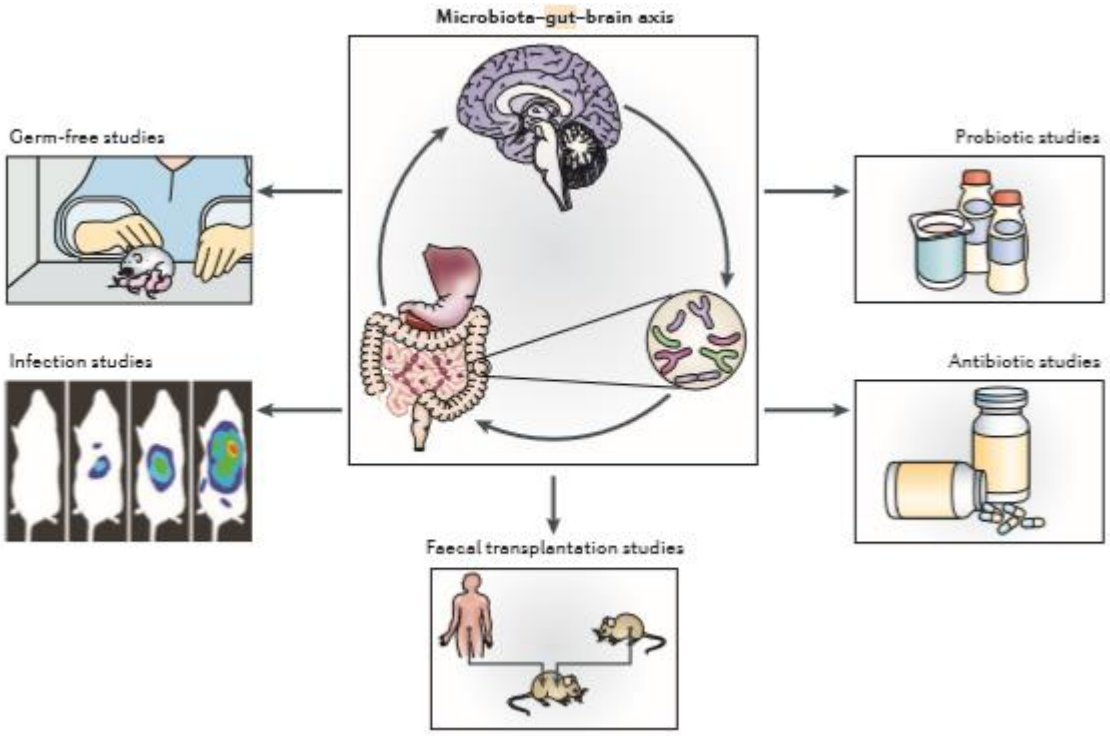


Figure 8. The microbiota-gut-brain axis is studied through a variety of methods.[40]



One current research topic of interest is the possible role of the gut microbiome in connection with autism spectrum disorder (ASD). According to recent studies, children with ASD tend to exhibit an increased number of *Clostridia* species in their microbiomes. [41,42] Interestingly, the gut microbiome has also been elucidated to have a possible role in other diseases such as schizophrenia, Parkinson's disease, and Alzheimer's disease.[41] The occurrence of altered microbiomes in these diseases only enhances the excitement of the idea that gut microbes may be involved in other diseases that have not yet been observed. In adulthood, the microbiome is more diverse and less susceptible to change. However, factors such as change in diet, geography, and lifestyle may alter the adult gut microbiome, which may lead to changes in health.[41]

Probiotics

The term "psychobiotics" encompasses any substance that affects the bacteria-brain relationship, which includes probiotics, prebiotics, antibiotics, and antipsychotic drugs.[37] Psychobiotics influence the body physically and psychologically due to the existence of the brain-gut axis.[37] Generally speaking, probiotics are bacteria that produce beneficial effects on health when administered in adequate quantities, an amount which varies between individuals.[36,38]

Probiotic Strains Used Experimentally

There have been several studies, mostly preclinical, on the effects of probiotics on mood, stress, cognition, anxiety, and inflammation. Strains administered to test effects on mood are typically from the genera *Lactobacillus* or *Bifidobacterium*. Species used include *B. infantis*, *B. longum*, *L. rhamnosus*, and *L. helveticus*.[36,38,43,44] Medicinal antidepressant treatments for depression are generally effective, although consistent use increases the risk for adverse effects.[27] In addition, current antidepressant medications are limited, and only treat specific aspects of the depression, such as reduced levels of serotonin.[20]

A multi-species microbial "cocktail" is being proposed for treatment due to the fact that different probiotic species produce different beneficial chemicals. *Lactobacillus* and *Bifidobacterium* species produce gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter. *Candida*, *Streptococcus*, *Escherichia*, and *Enterococcus* species produce serotonin, a neurotransmitter involved in mood regulation. *Bacillus* species produce dopamine, a neurotransmitter involved in processes such as reward, motivation, and memory. *Bifidobacterium infantis* in particular has been shown to alter kynurenine concentrations in the body, thus altering inflammation.[40] *Lactobacillus* and *Bifidobacterium* are also known to produce

certain unsaturated fatty acids and short chain fatty acids (SCFAs) that support cognitive function, brain development, and reduce inflammation.[27] For this proposal, species only from genera *Bifidobacteria* will be used in order to limit inhibition that can arise from having differing strains. *Lactobacilli* has been shown to inhibit other probiotic species on a larger scale than *Bifidobacteria.*[45]

Genera Bifidobacteria

The *Bifidobacterium* genus contains over 50 different species, with new species emerging each year.[46] Generally speaking, members of *Bifidobacterium* contribute to metabolic function by breaking down monosaccharides.[46] These bacteria are found in small portions in the human gut microbiome, among many other microbial populations. However, they are found in larger quantities within the microbiomes of infants, most likely due to the presence of oligosaccharides in breast milk.[46] Studies show that *Bifidobacteria* contribute to defense against pathogens and reduce risk of infection through various mechanisms, including synthesis of organic acids and antibacterial peptides. Specifically, *Bifidobacteria* has been observed to reduce the presence of *Helicobacter pylori*, thus reducing risk of infection.[46] Notably, members of *Bifidobacteria* may also benefit those with colorectal cancer, diarrhea, necrotizing entercolitis, IBS, and liver disease.[46,47]

PROBIOTIC ADMINISTRATION AS TREATMENT FOR HYPOTHYROID SYMPTOMS

Biochemical interactions and pathways involved in emotion and cognition

Psychophysiological effects of microbiota on the brain include modulation of inflammatory and stress processes, changes in emotional and cognitive responses, and production or reduction of neurotransmitters and proteins. Pathogens cause the immune system to release toxins and inflammatory cytokines into the body, which then cause adverse neurochemical changes. Probiotics have the ability to exclude pathogens through inhibition, interfere with signal transduction pathways, and produce antagonists which bind to inflammatory cytokines and prevent normal inflammatory responses.[27,48] Inflammation in the CNS is associated with negative psychological health, including depression, anxiety, and stress. Probiotics also have roles in lipid metabolism and maintaining tight junctions to prevent permeability in the blood-brain barrier (BBB). The gut-microbiome-brain (GMB) connection, which is the idea of complex interconnectedness and biochemical communication between such parts of the body, is important in understanding how probiotics affect mood.[27]

The tryptophan kynurenine pathway is a mechanism also involved in the brain-gut axis. Tryptophan is an amino acid that is a precursor for serotonin production. Probiotics produce several neural substances that are helpful in improving behavior. Thus, their role as therapy for neural disorders is promising.[49] Manipulation of tryptophan, and consequently serotonin levels, affect mood and cognition, which includes learning and memory. In general, lower serotonin levels in the brain correlate with worse memory and more depressed mood.[50] One study showed that depletion of tryptophan in non-depressed individuals had little effect on mood, while in depressed individuals, mood worsened. Similar results were observed in studies done in rats.

Additionally, it was suggested that tryptophan increases sleepiness, thus improving quality of sleep in depressed individuals who tend to suffer from lack of good sleep.[50] Certain microbiota, such as lactic acid bacteria, are able to metabolize tryptophan. Overall, increased consumption of foods that contain tryptophan will increase serotonin levels and improve mood in depressed individuals.[50] In addition, probiotics can help regulate the hypothalamic-pituitary-adrenal (HPA) axis, thereby reducing stress which is a factor of both anxiety and depression.[51]

The gastrointestinal microbiota also influence the presence of brain-derived neurotrophic factor (BDNF), which is a neurotrophic protein. Gut bacteria may control how BDNF functions in the CNS by causing changes in function of neurotransmitters and SCFAs.[49] Abnormal levels of hippocampal BDNF have been connected with weakening of synaptic transmissions and plasticity, thus resulting in cognitive impairments.[49] BDNF signaling occurs through the binding of two isoforms (m-BDNF and pro-BDNF) to two different neuronal membrane-bound receptors known as tyrosine kinase receptor (TrkB) and p75.[52,53] m-BDNF binding to TrkB is associated with LTP, while pro-BDNF binding to p75 is associated with LTD.[52,54] m-BDNF aids in development of neurons and glial cells, sustains strength of synapses, and decreases excitability of inhibitory GABA interneurons, functions that all contribute to LTP and memory persistence.[54–56] BDNF appears to be closely linked with the presence of N-methyl-D-aspartate (NMDA) glutamate receptors.[49] NMDA receptors are present on pre- and post-synaptic sites on neurons. They are involved in forming, controlling, and strengthening synaptic communications and modifying neurons.[49,55] BDNF modulation affects the function of NMDA receptors. Specifically, BDNF interacts with NMDA receptors to increase excitatory synaptic transmission of neurons. Furthermore, NMDA receptors support synthesis of BDNF.[49,57] Overall, deficits in

BDNF may lead to dysfunction of NMDA glutamate receptors, which is associated with depression and cognitive impairments.[49]

Hypothyroidism in rats has been shown to cause variations in BDNF, death of neurons and astrocytes, gliosis, and worsening of postsynaptic density.[58] Astrocytes are important in maintaining balance of ionic K^+ in the extracellular space, as well as in recycling glutamate and GABA. These roles are crucial in plasticity and the transmission of synapses.[59,60] In addition, astrocytes express many receptors that could be involved in memory and learning, including AMPA, NMDA, GABA, BDNF, serotonin, and IL-1β.[59] In one study, human glial progenitor cells were engrafted into the brains of neonatal immunodeficient mice. As the mice developed, they gained large amounts of human glial progenitors and astrocytes. It was shown that LTP and learning were increased compared to controls.[59,61] Thus, analysis of astrocytes may be important in recognizing cognitive deficits in humans and rodent models of hypothyroidism.

Animal Models Used Experimentally

Rodents are commonly used in probiotic preclinical trials and experiments, including models of Sprague-Dawley rats, Wistar rats, and neonatal or adult mice. Due to genetic differences in different strains and species of rodents, probiotics of the same species often have different effects depending on the rodent model used. There are some cases where beneficial probiotic effects are observed in one study, but no effects are observed in a subsequent study.[62]

A variety of tests exist to measure certain behaviors in rodents. The open field test examines anxious behaviors by measuring how long the rodent spends time away from a marked, center space. The forced swim test (FST) is used to measure depressive behaviors by observing how much rodents struggle to swim around in an enclosed pool of water.[37,63] Swimming, climbing, and immobility may be observed, and longer periods of immobility generally indicate

depressive behavior.[64] Maze-learning tasks, such as the Barnes maze and Morris water maze, are generally used to examine changes in memory, spatial learning, and anxious behaviors (*Figure 9*).[37] Fear conditioning and object recognition tests are used to test nonspatial learning and memory (*Figure 10*).[37]

Figure 9. Barnes maze.[88,89]

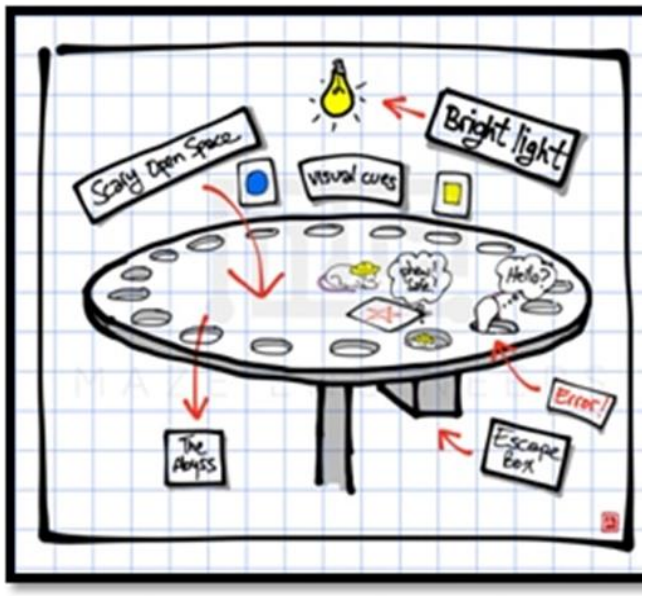
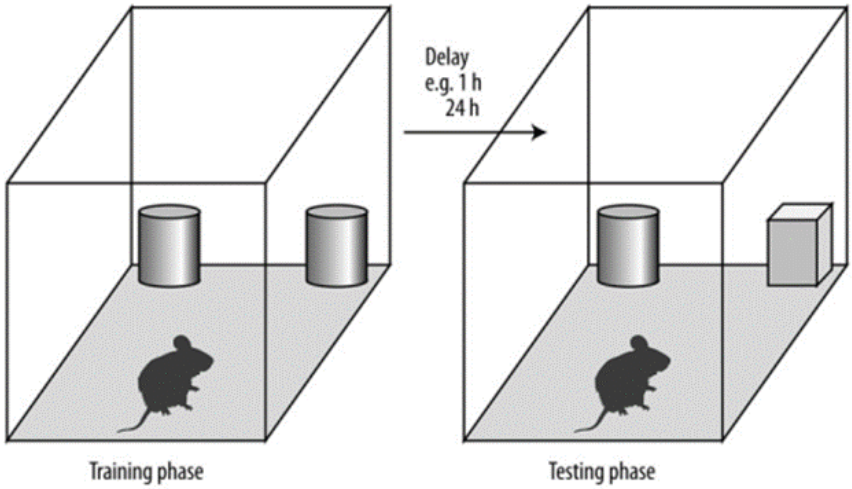


Figure 10. Object recognition test.[90]



Relevant Literature: Preclinical and Clinical Probiotic Studies

Bravo et al. 2011 conducted a preclinical study to observe the effects of *Lactobacillus rhamnosus* administration on neurotransmitter receptors in the CNS, paying special attention to GABA receptors.[65] The probiotic impacts different regions of the brain, causing an increase of GABA in cortical regions and a decrease in the amygdala and hippocampus. Overall, the probiotic reduced stress and anxiety-like behaviors in mice.[65] Effects in vagatomized mice, where the vagus nerve was inhibited, were not present. This aspect of the experiment emphasizes the vagus nerve's role in gut-brain communication.[65]

A preclinical study done by Desbonnet et al. 2008 investigated the effects of administration of the probiotic *Bifidobacteria infantis* on normal rats over a 14-day period. Effects were measured by observing behavior of rats in a FST, as well as by examining levels of cytokines, tryptophan, kynurenic acid, brain monoamines (dopamine, noradrenaline, and serotonin), AVP mRNA, CRF mRNA, and corticosterone.[66] There was no significant difference found between behaviors of control subjects versus probiotic subjects during the FST. This result could be due to the lack of stress in the subjects. It was found that *Bifidobacteria* subjects suppressed the release of proinflammatory cytokines compared to controls. Tryptophan and kynurenic acid concentrations were increased in probiotic specimens. There were no significant effects found in other factors measured.[66] The study suggests that *Bifidobacteria*'s beneficial neurological effects only become present in models suffering from severe stress or other psychopathological diseases, a phenomenon known as the ceiling effect.[37,66] The authors conducted a follow-up study using the maternal separation model of depression.[66]

In a follow-up study, Desbonnet et al. 2010 investigated the effects of administration of the probiotic *Bifidobacteria infantis* on rats that had suffered early chronic stress through the

maternal separation model. The maternal separation (MS) model involves separation of infant rats from their mother(s), causing early stress that disrupts the brain and gut.[64] The rats were separated into the following groups: normal (not separated), MS, MS/probiotic, MS/antidepressant (citalopram). Starting at 50 days of age, through day 95, the rats were given their assigned treatments. Effects were measured in the same way as the previous study. In the FST, the probiotic and antidepressant reduced immobility in rats and increased swim time. There was no effect on tryptophan or kynurenic acid in any of the groups. Abnormalities caused by maternal separation were found to be normalized in the probiotic and antidepressant groups.[64] The authors state that it is a possibility that MS causes biological alterations which inhibit tryptophan production by *B. infantis*. The results showed how stress can alter the microbiome and brain function, as compared to the previous study in normal rats. It is also apparent that *B. infantis* have similar effects to antidepressants, demonstrated by observations in the FST.[64]

A study done by Cortés et al. 2012 observed various biomarkers in hypothyroid adult male Sprague-Dawley rats.[58] Hypothyroidism was induced by treatment with 6-propyl-2-thiouracil (PTU) for 20 days. Adult rats with hypothyroidism have displayed reduced neurons in the hippocampal brain region, as well as abnormal expression of mRNA that encodes for specific subunits of NMDA receptors and TrkBs.[58] This study also observed reduced thickness of post synaptic density in the brains of hypothyroid rats, higher BDNF levels in their telencephalon regions, higher levels of BDNF mRNA in the hippocampal regions, and increased astrocyte death. This study provided evidence that hypothyroidism may be damaging to the hippocampal region of the brain.[58]

Davari et al. 2013 used probiotics to treat cognitive and synaptic impairment in diabetic-induced rats.[67] Oxidative stress is a primary mechanism that contributes to diabetic symptoms.

Thus, damage by oxidative stress is also a contributor to cognitive dysfunction in the disease.[67] Rats were administered a probiotic mixture of *Lactobacillus acidophilus*, *Bifidobacterium lactis*, and *Lactobacillus fermentum* dissolved in drinking water for two months. Spatial learning was assessed using the Morris water maze and excitatory postsynaptic potentials were measured using electrophysiological recordings. Overall, it was shown that probiotic administration improved spatial learning and synaptic plasticity in diabetic rats.[67] Notably, oxidative stress may also be involved in damaging brain tissue and contributing to cognitive impairment in hypothyroidism.[68]

In a controlled, randomized clinical trial conducted by Steenbergen et al. 2015, the effects of probiotics on cognitive reactivity were tested. Cognitive reactivity is triggered when mood changes occur, causing irregular thoughts such as those of hopelessness and aggression.[69] The objective of the experiment was to determine if the administering of probiotics reduces cognitive reactivity in healthy, young adults who have not been diagnosed with a mood disorder. 40 individuals were tested, with 20 in the experimental group and 20 in the placebo group. A triple-blind design was used, meaning the administer, participants, and assessor did not know which samples the placebos were. The probiotic sample contained seven different gut bacteria species: *B. bifidum, B. lactis, L. acidophilus, L. brevis, L. casei, L. salivarius,* and *L. lactis*. The Leiden Index of Depression Sensitivity was used to measure changes in participants' moods over a four-week period. The objective was accomplished by showing that overall cognitive reactivity was reduced through the ingestion of probiotics.[69] However, there were limitations which included an unequal ratio of female to male participants, unmonitored diets of participants, and self-administration of the samples. Regardless, the general effect of probiotics on mood in the

experimental group supports the idea that probiotics may be effective in treating mood disorders.[69]

Furthermore, Aizawa et al. 2016 investigated the bacteria count of *Bifidobacterium* and *Lactobacillus* in patients with Major Depressive Disorder (MDD) compared to healthy controls.[44] The results showed a significantly lower amount of Bifidobacterium in subjects with MDD. The amount of *Lactobacillus* in MDD patients was lower, but not as significant as with Bifidobacterium. It was also found that IBS was more common in patients with MDD. The study also noted that consumption of fermented milk correlated with higher levels of *Bifidobacterium* in subjects.[44] Depression was measured using the Hamilton depression rating scale. IBS was measured using the ROME III criteria. Fecal samples were used to measure the number of bacteria in the gut. A similar number of patients versus healthy subjects were used.[44] There were limitations in that that gender and diet were not completely taken into account. However, the results showed no significant differences due to these limitations. The study offers support for the helpful nature of specific probiotics in treating depression.[44]

In a clinical trial, Romijn et al. 2017 studied the effects of *Lactobacillus helveticus* and *Bifidobacterium longum* on individuals suffering from severe MDD symptoms.[70] 79 randomly selected individuals participated, with 40 being in the probiotic group and 39 being in the placebo group. Probiotics were administered for 8 weeks, and a variety of baseline tests were done. BDNF, IBS symptoms, vitamin D, and levels of proinflammatory cytokines were also measured due to their connectedness with depression. The results showed no evidence for significant improvement in mood or moderation of inflammation levels after administration of the specific probiotic mixture.[70] It is suggested that differences in results from other probiotic studies could be due to sample size, length of the trial, the severity or treatment resistance of

participants, or the bacterial strains used. Another explanation is that the specific probiotics used are only effective on low mood individuals, rather than MDD individuals. It is important to note that this particular study should not be generalized to all probiotic usage.[70]

Because of the success in many studies involving probiotics as treatment for various health problems, it is proposed that their use be studied on patients with thyroid dysfunction.

RESEARCH PROPOSAL

Observing the therapeutic effects of probiotics on impaired memory in hypothyroidism

The following probiotic experiment is adapted from studies done by Davari et al., Savignac et al., and Cortés et al.[58,71,72]

Experimental Procedure:

An animal model for hypothyroidism will be created using adult (7-8 weeks old), male Sprague-Dawley rats. To induce hypothyroidism, specimens will be treated with 0.05% of 6-propyl-2-thiouracil (PTU) in drinking water for a total of 20 days.[58] The control group will contain 10 rats with no sort of treatment. A second group will contain 10 rats treated with PTU. A third group will contain 10 rats administered only probiotics and no PTU. A fourth group will contain 10 rats administered both probiotics and PTU (*Table 2*). To measure levels of T_3, T_4, and TSH, serum levels of both hypothyroid (PTU) and control rats will be analyzed.[58] Chemioluminescence will be used to measure serum levels of T_3 and T_4, while radioimmunoassay will be used to measure TSH.[58]

Table 2. Experimental groups.

Group	(n)
No treatment (control)	10
PTU-only	10
Probiotic-only	10
PTU and probiotic	10

The probiotic mixture will contain the strains *Bifidobacterium longum*, *Bifidobacterium breve*, and *Bifidobacterium infantis* at a quantity of $1x10^9$ CFU/g. 1 g of the freeze-dried mixture will be dissolved in drinking water of each mouse once every 12 hours.[71,72] Drinking water intake will be measured in all groups to ensure equal consumption. Rats will be fed for 11 weeks total before sacrifice and behavioral testing will begin at 3 weeks.[72]

The following behavioral tests will be done to measure memory and learning (*Table 3*):

Table 3. Measurements for memory

Barnes Maze (*Figure 9*)	The Barnes Maze is a test for spatial memory. It involves an elevated circular platform with 18 holes evenly spaced along the circumference. Visual cues are placed around the room to help guide the rat to an escape hole. A bright light is placed above the platform, which drives a rat's natural tendency to avoid bright lights and find an escape.[73] Preliminary training will be done on day 1 by guiding each rat using a treat, and testing will be done 15 minutes after training and on days 3, 10, 24, 45, and 52.
Object Recognition (*Figure 10*)	Object recognition, also known as the novel object test, evaluates non-spatial learning and memory.[72,74,75] It demonstrates function of the dorsal hippocampus and is based on a rat's tendency to explore novel objects.[74,76] The protocol involves three phases. The habituation phase allows rats to explore an open arena in the absence of any objects. The familiarization phase allows them to explore two identical novel objects in the arena. The testing phase replaces one of the objects with a different one and observes the rat's tendency to explore novel objects over familiar ones. In this experiment testing will be held in week 8 of testing. Each phase will last 10 minutes. The habituation phase will occur on the first day, the familiarization phase will occur on the second day, and the testing phase will occur on the third day. Memory will be measured as time spent exploring the novel object

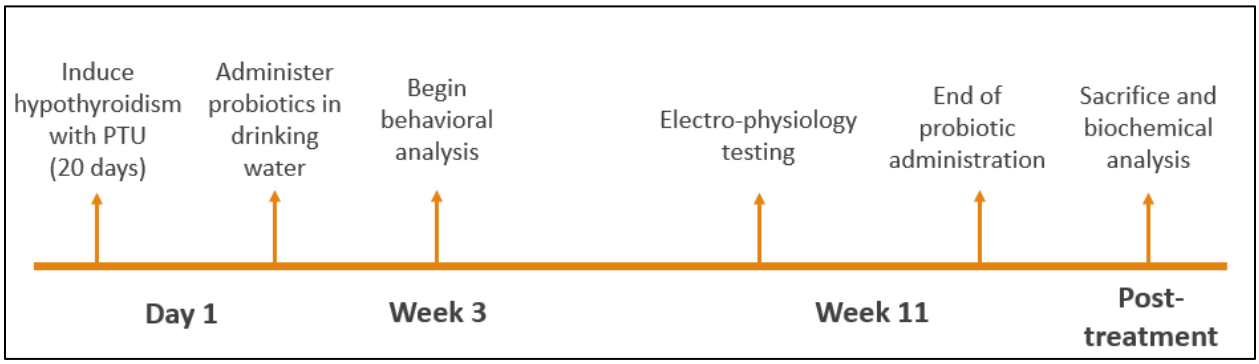
	over the familiar one. Rats with better memory will explore the novel object with a ratio greater than 50%.[72,76]
Fear Conditioning	The fear-conditioning test associates a conditional stimulus (CS) with an unconditional stimulus (US). The CS will be a light cue while the US will be a foot shock. When a foot shock is associated with a CS, rats will learn to fear that stimulus. Fear translates as freezing, or absence of movement.[72] Fear conditioning will occur during week 7 of testing. The procedure will last 3 consecutive days total and will include a day of training (learning) and two days of testing (memory assessment).[72] On the first day, rats will be presented both the CS and US. On the next two days, only the CS will be presented.[72] Rats that have more freezing time are associated with better learning and memory since they are able to better remember the presence of a shock from training.

In week 8 of testing, electrophysiological recordings will be done in the hippocampal CA3-CA1 pathways. Rats will be anesthetized, and two holes will be drilled into their skulls for the stimulating and recording electrodes to enter.[71] Excitatory postsynaptic potential (EPSPs) will be recorded at baseline rates. Then, high-frequency stimulation of 100 Hz will be used to induce an LTP response. Mean amplitudes will be calculated for pre-stimulation, basal EPSPs and high-frequency stimulated EPSPs.[71]

After sacrifice (8 weeks), a variety of biomarkers will be tested. Apoptosis of hippocampal neurons and astrocytes will be analyzed using confocal microscopy. BDNF will be

measured by using in situ hybridization and enzyme-linked immunosorbent assay (ELISA).[58] Electron microscopy will be used to observe glutamergic synapse and postsynaptic density (PSD).[58] Western blot will be used to analyze PSD proteins such as NMDAR, TrkB, and p75.

Figure 11. Timeline for first experimental procedure



Expected Outcomes

It is predicted that the control group and probiotic-fed groups will perform better in the Barnes maze than the PTU-only group. It is likely that the control and probiotic-fed groups will have similar performances. In the object recognition test, the control group and probiotic-fed groups will spend more time near the novel object than the familiar object, while PTU-only rats will spend similar amounts of time near both objects. In the fear-conditioning test, the control group and probiotic-fed groups will have higher percentages of freezing time than the PTU-only group. In the electrophysiological tests of the hippocampus, it is predicted that basal rates will be similar in all groups. However, high-frequency stimulation will produce differences in LTPs. PTU-only rats will have significantly decreased potentiation compared to control and probiotic-fed rats. Biomarkers for BDNF will be higher in PTU-only rats than in controls or probiotic-fed. In addition, NMDA and TrkB receptors will be lower in PTU-only rats than in other groups, and p75 will remain similar throughout all groups. PSD will be thinner in PTU-only rats than in other groups. Astrocyte and neuronal apoptosis will be significantly higher in PTU-only rats than in

other groups. It is important to note that rats in the PTU-probiotic are still lacking thyroid hormone. Thus, it is predicted that learning and memory behaviors, LTPs, and presence of certain biomarkers will be less than the control and probiotic-only groups. However, results for the PTU-probiotic group are projected to still be better than the PTU-only group.

Observing the therapeutic effects of probiotics on depressive and anxiety symptoms in hypothyroidism

In some hypothyroid patients who take L-thyroxine (T), reversal of anxiety and depressive symptoms is observed. However, there are also cases where mood symptoms persist even after thyroxine administration. Thus, a secondary treatment involving antidepressants and/or anxiolytics may be necessary to properly treat depression and anxiety symptoms in hypothyroid patients.[4,77,78] The proposed clinical trial suggests use of a multi-strain *Bifidobacteria* probiotic mixture as treatment for depression and anxiety in place of typical antidepressant and anxiolytic drugs.

Experimental Procedure:

The following double-blind, randomized controlled clinical trial procedure is adapted from two trials done by Romijn et al and Steenbergen et al.[69,70]

A total of 80 participants will be screened from adult (18+) hypothyroid patients with minor to severe depression and anxiety. Participants will have been taking L-thyroxine for at least 6 months prior to the start of the experiment. Inclusion criteria will be as follows: (1) either $\geqslant$11 on the Quick Inventory of Depressive Symptomatology (QIDS-SR16) or $\geqslant$14 on the depression subscale of the Depression, Anxiety and Stress Scale (DASS-42); (2) aged 18+ at the time of screening; and (3) free of any psychiatric medication for at least 4 weeks prior to the trial.[70] Demographics including sex, date of birth, ethnicity, household income, marital status,

occupation, and highest educational qualification will be obtained.[70] Participants will not be able to take any antidepressants, anxiolytics, or other probiotic supplements during the trial. Additional exclusion criteria will be as follows: any neurological disorder, any serious medical condition that may require medical intervention during the trial, renal, hepatic, cardiovascular or respiratory disease, any pregnant or breastfeeding woman, serious suicide risk or risk of violence, use of any supplement with potentially antidepressant properties, any recent or current use of probiotics or antibiotics.[70]

Scales for depression and anxiety will be administered as follows (*Table 4*):

Table 4. Measurements for anxiety and depression

Beck Anxiety Inventory	This scale is a 21-item self-rating questionnaire for measuring severity of anxiety.[79,80] Scores range from 0-63, with scores less than 9 being normal and scores over 30 being very severe.[69] Participants will be rated at baseline and every 2 weeks after for 8 weeks.
16-Item quick inventory of depressive symptomatology self-report	The QIDS-SR16 is used to measure severity of depression.[81,82] It is scored on a scale of 0-27, where a score less than 6 is rated as none and a score over 20 is rated as very severe.[70] Participants will be rated at baseline and every 2 weeks after for 8 weeks.

Montgomery–Åsberg Depression Rating Scale	The MADRS is a questionnaire used to measure severity of depression on a 60-point scale, where a score higher than 35 is rated as severe and a score below 8 is rated as none. It contains 10 items and is broadly accepted for its sensitivity to change.[83] Thus, it will be useful in measuring effects of treatment over the time of the trial.[70] Participants will be rated at baseline, 4 weeks, and 8 weeks.
DASS-42	This scale is a self-report questionnaire that contains 42 items. It is used to measure symptoms of depression, anxiety, and stress. Participants will be rated at baseline, at 4 weeks, and at 8 weeks.[70]

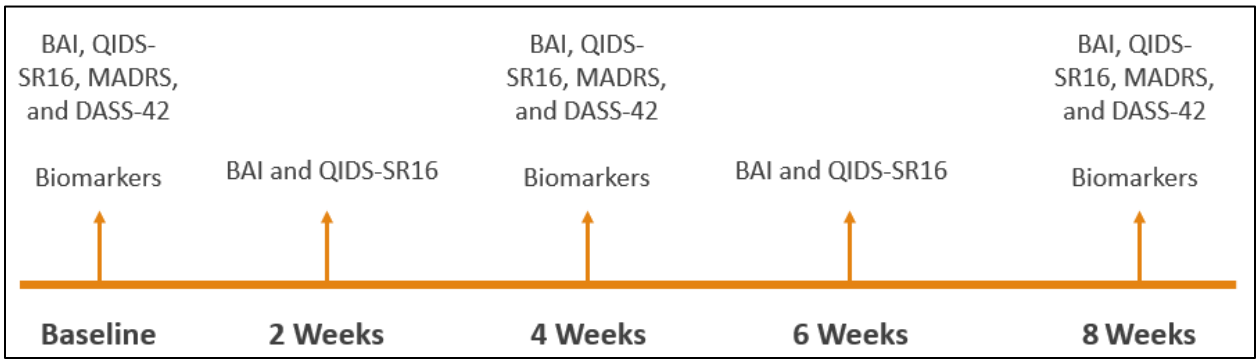
The probiotic treatment will be administered in the form of sachets containing 2g of freeze-dried powder of a mixture containing the species *Bifidobacterium longum*, *Bifidobacterium breve*, and *Bifidobacterium infantis* (2.5×10^9 CFU/g).[69]
The placebo sachets will contain 2g of freeze-dried powder containing maize starch and maltodextrins.[69] It is imperative that the placebo has the same taste, color, and smell as the probiotic in order for the double-blind trial to be effective.

The 80 participants will be randomly sorted into a placebo or probiotic group containing 40 participants each. Participants will be given 4-weeks' worth of treatment at a time. At 4 weeks, participants will come to the lab for blood work, clinically-administered questionnaires, and to receive the last 4-weeks' worth of treatment. They will be instructed to consume one sachet at the same time every day for 8-weeks by drinking the powder via water or milk. A reminder will be sent through text.[69]

Participants will be asked to log foods eaten, substances they drink (especially concerning caffeine and alcohol), and other habits that may affect results (nicotine or drug use). In addition, self-reports will be done every 2 weeks and participants will be asked to note any missed doses.[70]

Biomarkers will be measured at baseline, 4 weeks, and 8 weeks. Blood samples and ELISA immunoassays will be used to measure levels of high-sensitivity C-reactive protein (hsCRP), IL-1β, IL-6, TNF-α, and BDNF. BDNF is involved in cognitive flexibility and hsCRP, IL-1β, IL-6, and TNF-α are biomarkers for inflammation.[70,84] Urine testing for cortisol, a stress hormone, will be done.[85] In addition, serum TSH levels will be measured at baseline, 4 weeks, and 8 weeks to ensure there is no significant variance.[77]

Figure 12. Timeline for second experimental procedure



Expected Outcomes

It is predicted that participants administered the probiotic treatment will have lower scores on the depression/anxiety scales at 8 weeks compared to their baseline scores than those in the placebo group. Biomarkers for inflammation cortisol, and BDNF will be lower in the probiotic group than in the placebo. Serum TSH levels should remain the same for all participants. Results could be compared to other studies that have used antidepressants and anxiolytics.

CONCLUSIONS, LIMITATIONS, AND FUTURE AIMS

Based on studies done previously in models of depression, anxiety, and cognition, it is anticipated that probiotic administration will help reduce cognitive and psychiatric symptoms, to antidepressants and other methods of treatment.

Since previous studies have varied in their results, future research should attempt to observe probiotic effects on different species or strains of rats, as well as different methods of delivery. There is no certainty these probiotic strains will have the desired effects in humans if treatment is only successful in one strain of rats. Additionally, future research should explore different multi-species combinations or single strain administration as treatment. Both preclinical and clinical studies have produced varying results depending on the types of probiotics being administered. Some data suggests that multi-strain mixtures may be less efficient due to increased competition between species. Other data suggests that multi-strain mixtures are more efficient because of the larger concentration of bacteria present and a wider range of effects produced.[86] This contradictory information should therefore be addressed in future work. Additionally, research involving administration of prebiotics alongside probiotics should be done to observe if beneficial effects of probiotics could be increased with prebiotics. Finally, the proposed studies could be repeated with additional groups being administered antidepressants. Thus, effects of antidepressants and probiotics in hypothyroid models could be studied alongside each other. Effects of combined administration of both antidepressants and probiotics could also be observed.

Research in the field of probiotics is novel, and much is unknown as to how certain effects are produced by microbes via the gut-brain axis. Variations in results occur depending on strains of bacteria and rodents, making it difficult to translate successful preclinical results in

human models. As more researchers become attracted by the intriguing phenomena produced by probiotics, the field will greatly increase in knowledge. Until then, research should be done to increase knowledge of mechanisms involved in depression, anxiety, and memory, which will better help identify which probiotics may alter those mechanisms.

REFERENCES

1. Preety R, M. S. T. Hypothyroidism and its implications among women. *Drug Invent Today*. 2018;10(11):2330-2334. https://seu.idm.oclc.org/login?url=https://search.ebscohost.com/login.aspx?direct=true&db=a9h&AN=132173510&site=ehost-live&scope=site. Accessed January 23, 2019.

2. Gaitonde DY, Rowley KD, Sweeney LB. Hypothyroidism: An Update. *Am Fam Physician*. 2012;86(3):244-251. https://www.aafp.org/afp/2012/0801/p244.html. Accessed March 10, 2019.

3. Hypothyroidism (Underactive Thyroid) | NIDDK. National Institute of Diabetes and Digestive and Kidney Diseases. https://www.niddk.nih.gov/health-information/endocrine-diseases/hypothyroidism. Accessed October 27, 2018.

4. Samuels MH. Psychiatric and cognitive manifestations of hypothyroidism. *Curr Opin Endocrinol Diabetes Obes*. 2014;21(5):377-383. doi:10.1097/MED.0000000000000089

5. Heinrich TW, Grahm G. Hypothyroidism Presenting as Psychosis: Myxedema Madness Revisited. *Prim Care Companion J Clin Psychiatry*. 2003;5(6):260-266. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC419396/. Accessed March 13, 2019.

6. Marieb E, Hoehn K. *Human Anatomy and Physiology*. Pearson; 2016.

7. Zoeller RT, Tan SW, Tyl RW. General Background on the Hypothalamic-Pituitary-Thyroid (HPT) Axis. *Crit Rev Toxicol*. 2007;37(1-2):11-53. doi:10.1080/10408440601123446

8. Carvalho DP, Dupuy C. Thyroid hormone biosynthesis and release. *Mol Cell Endocrinol*. 2017;458:6-15. doi:10.1016/j.mce.2017.01.038

9. Ortiga-Carvalho TM, Chiamolera MI, Pazos-Moura CC, Wondisford FE. Hypothalamus-Pituitary-Thyroid Axis. *Compr Physiol*. 2016;6(3):1387-1428. doi:10.1002/cphy.c150027

10. Deiodinases. *Natl Acad Hypothyroidism*. https://www.nahypothyroidism.org/deiodinases/. Accessed January 27, 2019.

11. Rivas M, Naranjo JR. Thyroid hormones, learning and memory. *Genes Brain Behav*. 2007;6(s1):40-44. doi:10.1111/j.1601-183X.2007.00321.x

12. De Leo S, Lee SY, Braverman LE. Hyperthyroidism. *Lancet Lond Engl*. 2016;388(10047):906-918. doi:10.1016/S0140-6736(16)00278-6

13. Kravets I. Hyperthyroidism: Diagnosis and Treatment. *Am Fam Physician*. 2016;93(5):363-370.

14. Osuna PM, Udovcic M, Sharma MD. Hyperthyroidism and the Heart. *Methodist DeBakey Cardiovasc J*. 2017;13(2):60-63. doi:10.14797/mdcj-13-2-60

15. Hyperthyroidism. *Am Thyroid Assoc*. https://www.thyroid.org/hyperthyroidism/. Accessed October 29, 2018.

16. Pyzik A, Grywalska E, Matyjaszek-Matuszek B, Roliński J. Immune disorders in Hashimoto's thyroiditis: what do we know so far? *J Immunol Res*. 2015;2015:979167. doi:10.1155/2015/979167

17. Rastogi MV, LaFranchi SH. Congenital hypothyroidism. *Orphanet J Rare Dis*. 2010;5:17. doi:10.1186/1750-1172-5-17

18. Krysiak R, Szkróbka W, Okopień B. Sexual function and depressive symptoms in young women with hypothyroidism receiving levothyroxine/liothyronine combination therapy: a pilot study. *Curr Med Res Opin*. 2018;34(9):1579-1586. doi:10.1080/03007995.2018.1448771

19. Krysiak R, Marek B, Okopień B. Sexual function and depressive symptoms in young men with hypothyroidism receiving levothyroxine/liothyronine combination therapy. *Pol J Endocrinol Endokrynol Pol*. 2018;69(1):16-22. doi:10.5603/EP.a2018.0005

20. Dean J, Keshavan M. The neurobiology of depression: An integrated view. *Asian J Psychiatry*. 2017;27:101-111. doi:10.1016/j.ajp.2017.01.025

21. Fischer S, Ehlert U. Hypothalamic–pituitary–thyroid (HPT) axis functioning in anxiety disorders. A systematic review. *Depress Anxiety*. 2018;35(1):98-110. doi:10.1002/da.22692

22. Effect of Probiotics on Depression: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4997396/. Accessed March 17, 2018.

23. Gerges NZ, Alkadhi KA. Hypothyroidism impairs late LTP in CA1 region but not in dentate gyrus of the intact rat hippocampus: MAPK involvement. *Hippocampus*. 2004;14(1):40-45. doi:10.1002/hipo.10165

24. Franklin AV, King MK, Palomo V, Martinez A, McMahon LL, Jope RS. Glycogen synthase kinase-3 inhibitors reverse deficits in long-term potentiation and cognition in Fragile X mice. *Biol Psychiatry*. 2014;75(3). doi:10.1016/j.biopsych.2013.08.003

25. Singh R, Tandon A, Gupta SK, Saroja K. Optimal Levothyroxine Replacement Adequately Improves Symptoms of Hypothyroidism; Residual Symptoms Need Further Evaluation for Other than Hypothyroidism Causation. *Indian J Endocrinol Metab*. 2017;21(6):830-835. doi:10.4103/ijem.IJEM_165_17

26. Wekking EM, Appelhof BC, Fliers E, et al. Cognitive functioning and well-being in euthyroid patients on thyroxine replacement therapy for primary hypothyroidism. *Eur J Endocrinol*. 2005;153(6):747-753. doi:10.1530/eje.1.02025

27. Gayathri D, Rashmi BS. Mechanism of development of depression and probiotics as adjuvant therapy for its prevention and management. *Ment Health Prev*. 2017;5:40-51. doi:10.1016/j.mhp.2017.01.003

28. Khawam EA, Laurencic G, Malone DA. Side effects of antidepressants: an overview. *Cleve Clin J Med*. 2006;73(4):351-353. doi:10.3949/ccjm.73.4.351

29. Buras A, Battle L, Landers E, Nguyen T, Vasudevan N. Thyroid hormones regulate anxiety in the male mouse. *Horm Behav*. 2014;65(2):88-96. doi:10.1016/j.yhbeh.2013.11.008

30. Özgür E, Gürbüz Özgür B, Aksu H, Cesur G. The Effect of Congenital and Postnatal Hypothyroidism on Depression-Like Behaviors in Juvenile Rats. *J Clin Res Pediatr Endocrinol*. 2016;8(4):439-444. doi:10.4274/jcrpe.3498

31. Zeng H, Schimpf BA, Rohde AD, Pavlova MN, Gragerov A, Bergmann JE. Thyrotropin-Releasing Hormone Receptor 1-Deficient Mice Display Increased Depression and Anxiety-Like Behavior. *Mol Endocrinol*. 2007;21(11):2795-2804. doi:10.1210/me.2007-0048

32. Hage MP, Azar ST. The Link between Thyroid Function and Depression. *J Thyroid Res*. 2012;2012. doi:10.1155/2012/590648

33. Tayde PS, Bhagwat NM, Sharma P, et al. Hypothyroidism and Depression: Are Cytokines the Link? *Indian J Endocrinol Metab*. 2017;21(6):886-892. doi:10.4103/ijem.IJEM_265_17

34. Bathla M, Singh M, Relan P. Prevalence of anxiety and depressive symptoms among patients with hypothyroidism. *Indian J Endocrinol Metab*. 2016;20(4):468-474. doi:10.4103/2230-8210.183476

35. Yoo BB, Mazmanian SK. The Enteric Network: Interactions between the Immune and Nervous Systems of the Gut. *Immunity*. 2017;46(6):910-926. doi:10.1016/j.immuni.2017.05.011

36. Saulnier DM, Ringel Y, Heyman MB, et al. The intestinal microbiome, probiotics and prebiotics in neurogastroenterology. *Gut Microbes*. 2013;4(1):17-27. doi:10.4161/gmic.22973

37. Sarkar A, Lehto SM, Harty S, Dinan TG, Cryan JF, Burnet PWJ. Psychobiotics and the Manipulation of Bacteria–Gut–Brain Signals. *Trends Neurosci*. 2016;39(11):763-781. doi:10.1016/j.tins.2016.09.002

38. Wang H, Lee I-S, Braun C, Enck P. Effect of Probiotics on Central Nervous System Functions in Animals and Humans: A Systematic Review. *J Neurogastroenterol Motil*. 2016;22(4):589-605. doi:10.5056/jnm16018

39. Dinan TG, Stilling RM, Stanton C, Cryan JF. Collective unconscious: how gut microbes shape human behavior. *J Psychiatr Res*. 2015;63:1-9. doi:10.1016/j.jpsychires.2015.02.021

40. Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci*. 2012;13(10):701-712. doi:10.1038/nrn3346

41. Sharon G, Sampson TR, Geschwind DH, Mazmanian SK. The Central Nervous System and the Gut Microbiome. *Cell*. 2016;167(4):915-932. doi:10.1016/j.cell.2016.10.027

42. Vuong HE, Hsiao EY. Emerging Roles for the Gut Microbiome in Autism Spectrum Disorder. *Biol Psychiatry*. 2017;81(5):411-423. doi:10.1016/j.biopsych.2016.08.024

43. Ushakova G, Fed'kiv O, Prykhod'ko O, Pierzynowski S, Kruszewska D. The effect of long-term lactobacilli (lactic acid bacteria) enteral treatment on the central nervous system of growing rats. *J Nutr Biochem*. 2009;20(9):677-684. doi:10.1016/j.jnutbio.2008.06.010

44. Aizawa E, Tsuji H, Asahara T, et al. Possible association of Bifidobacterium and Lactobacillus in the gut microbiota of patients with major depressive disorder. *J Affect Disord*. 2016;202:254-257. doi:10.1016/j.jad.2016.05.038

45. Chapman CMC, Gibson GR, Rowland I. In vitro evaluation of single- and multi-strain probiotics: Inter-species inhibition between probiotic strains, and inhibition of pathogens. *Anaerobe*. 2012;18(4):405-413. doi:10.1016/j.anaerobe.2012.05.004

46. Hidalgo-Cantabrana C, Delgado S, Ruiz L, Ruas-Madiedo P, Sánchez B, Margolles A. Bifidobacteria and Their Health-Promoting Effects. *Microbiol Spectr*. 2017;5(3). doi:10.1128/microbiolspec.BAD-0010-2016

47. O'Callaghan A, van Sinderen D. Bifidobacteria and Their Role as Members of the Human Gut Microbiota. *Front Microbiol*. 2016;7. doi:10.3389/fmicb.2016.00925

48. Kobyliak N, Abenavoli L, Mykhalchyshyn G, et al. A Multi-strain Probiotic Reduces the Fatty Liver Index, Cytokines and Aminotransferase levels in NAFLD Patients: Evidence from a Randomized Clinical Trial. *J Gastrointestin Liver Dis*. 2018;27(1):41-49. doi:10.15403/jgld.2014.1121.271.kby

49. Maqsood R, Stone TW. The Gut-Brain Axis, BDNF, NMDA and CNS Disorders. *Neurochem Res*. 2016;41(11):2819-2835. doi:10.1007/s11064-016-2039-1

50. Jenkins TA, Nguyen JCD, Polglaze KE, Bertrand PP. Influence of Tryptophan and Serotonin on Mood and Cognition with a Possible Role of the Gut-Brain Axis. *Nutrients*. 2016;8(1). doi:10.3390/nu8010056

51. Mason BL. Feeding Systems and the Gut Microbiome: Gut-Brain Interactions With Relevance to Psychiatric Conditions. *Psychosomatics*. 2017;58(6):574-580. doi:10.1016/j.psym.2017.06.002

52. Castrén E, Rantamäki T. The role of BDNF and its receptors in depression and antidepressant drug action: Reactivation of developmental plasticity. *Dev Neurobiol*. 2010;70(5):289-297. doi:10.1002/dneu.20758

53. Hempstead BL. Brain-Derived Neurotrophic Factor: Three Ligands, Many Actions. *Trans Am Clin Climatol Assoc*. 2015;126:9-19.

54. Kowiański P, Lietzau G, Czuba E, Waśkow M, Steliga A, Moryś J. BDNF: A Key Factor with Multipotent Impact on Brain Signaling and Synaptic Plasticity. *Cell Mol Neurobiol*. 2018;38(3):579-593. doi:10.1007/s10571-017-0510-4

55. Phillips C. Brain-Derived Neurotrophic Factor, Depression, and Physical Activity: Making the Neuroplastic Connection. *Neural Plast*. 2017;2017. doi:10.1155/2017/7260130

56. Bekinschtein P, Cammarota M, Katche C, et al. BDNF is essential to promote persistence of long-term memory storage. *Proc Natl Acad Sci U S A*. 2008;105(7):2711-2716. doi:10.1073/pnas.0711863105

57. Crozier RA, Bi C, Han YR, Plummer MR. BDNF Modulation of NMDA Receptors Is Activity Dependent. *J Neurophysiol*. 2008;100(6):3264-3274. doi:10.1152/jn.90418.2008

58. Cortés C, Eugenin E, Aliaga E, et al. Hypothyroidism in the Adult Rat Causes Incremental Changes in Brain-Derived Neurotrophic Factor, Neuronal and Astrocyte Apoptosis, Gliosis, and Deterioration of Postsynaptic Density. *Thyroid*. 2012;22(9):951-963. doi:10.1089/thy.2010.0400

59. Moraga-Amaro R, Jerez-Baraona JM, Simon F, Stehberg J. Role of astrocytes in memory and psychiatric disorders. *J Physiol-Paris*. 2014;108(4):240-251. doi:10.1016/j.jphysparis.2014.08.005

60. Sofroniew MV, Vinters HV. Astrocytes: biology and pathology. *Acta Neuropathol (Berl)*. 2010;119(1):7-35. doi:10.1007/s00401-009-0619-8

61. Han X, Chen M, Wang F, et al. Forebrain engraftment by human glial progenitor cells enhances synaptic plasticity and learning in adult mice. *Cell Stem Cell*. 2013;12(3):342-353. doi:10.1016/j.stem.2012.12.015

62. Foster JA, McVey Neufeld K-A. Gut–brain axis: how the microbiome influences anxiety and depression. *Trends Neurosci*. 2013;36(5):305-312. doi:10.1016/j.tins.2013.01.005

63. Krishnan V, Nestler EJ. Animal Models of Depression: Molecular Perspectives. *Curr Top Behav Neurosci*. 2011;7:121-147. doi:10.1007/7854_2010_108

64. Desbonnet L, Garrett L, Clarke G, Kiely B, Cryan JF, Dinan TG. Effects of the probiotic Bifidobacterium infantis in the maternal separation model of depression. *Neuroscience*. 2010;170(4):1179-1188. doi:10.1016/j.neuroscience.2010.08.005

65. Bravo JA, Forsythe P, Chew MV, et al. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci U S A*. 2011;108(38):16050-16055. doi:10.1073/pnas.1102999108

66. Desbonnet L, Garrett L, Clarke G, Bienenstock J, Dinan TG. The probiotic Bifidobacteria infantis: An assessment of potential antidepressant properties in the rat. *J Psychiatr Res*. 2008;43(2):164-174. doi:10.1016/j.jpsychires.2008.03.009

67. Davari S, Talaei SA, Salami M, Fazeli MR, Alaei H. Effects of Supplementation with Probiotics on Long-Term Potentiation Impairment in Diabetic Rats. *J Isfahan Med Sch*. 2013;30(218):2236-2247. https://seu.idm.oclc.org/login?url=https://search.ebscohost.com/login.aspx?direct=true&db=a9h&AN=90069020&site=ehost-live&scope=site. Accessed October 27, 2018.

68. Farrokhi E, Hosseini M, Beheshti F, Vafaee F, Hadjzadeh MA-R, Dastgheib SS. Brain tissues oxidative damage as a possible mechanism of deleterious effects of propylthiouracil-induced hypothyroidism on learning and memory in neonatal and juvenile growth in rats. *Basic Clin Neurosci*. 2014;5(4):285-294.

69. Steenbergen L, Sellaro R, van Hemert S, Bosch JA, Colzato LS. A randomized controlled trial to test the effect of multispecies probiotics on cognitive reactivity to sad mood. *Brain Behav Immun*. 2015;48:258-264. doi:10.1016/j.bbi.2015.04.003

70. Romijn AR, Rucklidge JJ, Kuijer RG, Frampton C. A double-blind, randomized, placebo-controlled trial of Lactobacillus helveticus and Bifidobacterium longum for the symptoms of depression. *Aust N Z J Psychiatry*. 2017;51(8):810-821. doi:10.1177/0004867416686694

71. Davari S, Talaei SA, Alaei H, salami M. Probiotics treatment improves diabetes-induced impairment of synaptic activity and cognitive function: Behavioral and electrophysiological proofs for microbiome–gut–brain axis. *Neuroscience*. 2013;240:287-296. doi:10.1016/j.neuroscience.2013.02.055

72. Savignac HM, Tramullas M, Kiely B, Dinan TG, Cryan JF. Bifidobacteria modulate cognitive processes in an anxious mouse strain. *Behav Brain Res*. 2015;287:59-72. doi:10.1016/j.bbr.2015.02.044

73. Ohland CL, Kish L, Bell H, et al. Effects of Lactobacillus helveticus on murine behavior are dependent on diet and genotype and correlate with alterations in the gut microbiome. *Psychoneuroendocrinology*. 2013;38(9):1738-1747. doi:10.1016/j.psyneuen.2013.02.008

74. Gareau MG, Wine E, Rodrigues DM, et al. Bacterial infection causes stress-induced memory dysfunction in mice. *Gut*. 2011;60(3):307-317. doi:10.1136/gut.2009.202515

75. Antunes M, Biala G. The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cogn Process*. 2012;13(2):93-110. doi:10.1007/s10339-011-0430-z

76. Smith CJ, Emge JR, Berzins K, et al. Probiotics normalize the gut-brain-microbiota axis in immunodeficient mice. *Am J Physiol - Gastrointest Liver Physiol*. 2014;307(8):G793-G802. doi:10.1152/ajpgi.00238.2014

77. Panicker V, Evans J, Bjøro T, Asvold BO, Dayan CM, Bjerkeset O. A paradoxical difference in relationship between anxiety, depression and thyroid function in subjects on

and not on T4: findings from the HUNT study. *Clin Endocrinol (Oxf)*. 2009;71(4):574-580. doi:10.1111/j.1365-2265.2008.03521.x

78. Saravanan P, Chau W-F, Roberts N, Vedhara K, Greenwood R, Dayan CM. Psychological well-being in patients on 'adequate' doses of l-thyroxine: results of a large, controlled community-based questionnaire study. *Clin Endocrinol (Oxf)*. 2002;57(5):577-585. doi:10.1046/j.1365-2265.2002.01654.x

79. Beck AT, Epstein N, Brown G, Steer RA. An inventory for measuring clinical anxiety: Psychometric properties. *J Consult Clin Psychol*. 1988;56(6):893-897. doi:10.1037/0022-006X.56.6.893

80. Muntingh AD, van der Feltz-Cornelis CM, van Marwijk HW, Spinhoven P, Penninx BW, van Balkom AJ. Is the beck anxiety inventory a good tool to assess the severity of anxiety? A primary care study in The Netherlands study of depression and anxiety (NESDA). *BMC Fam Pract*. 2011;12:66. doi:10.1186/1471-2296-12-66

81. Brown ES, Murray M, Carmody TJ, et al. The Quick Inventory of Depressive Symptomatology–Self-report: a psychometric evaluation in patients with asthma and major depressive disorder. *Ann Allergy Asthma Immunol Off Publ Am Coll Allergy Asthma Immunol*. 2008;100(5):433-438. doi:10.1016/S1081-1206(10)60467-X

82. Rush AJ, Trivedi MH, Ibrahim HM, et al. The 16-Item quick inventory of depressive symptomatology (QIDS), clinician rating (QIDS-C), and self-report (QIDS-SR): a psychometric evaluation in patients with chronic major depression. *Biol Psychiatry*. 2003;54(5):573-583. doi:10.1016/S0006-3223(02)01866-8

83. Montgomery SA, Asberg M. A new depression scale designed to be sensitive to change. *Br J Psychiatry J Ment Sci*. 1979;134:382-389.

84. Kostić-Petrović I, Žikić O. Biomarkers of Depression: New Challenges. *BIOMARKERI DEPRESIJE- NOVI IZAZOVI*. 2017;56(1):44-49. doi:10.5633/amm.2017.0107

85. Messaoudi M, Lalonde R, Violle N, et al. Assessment of psychotropic-like properties of a probiotic formulation (Lactobacillus helveticus R0052 and Bifidobacterium longum R0175) in rats and human subjects. *Br J Nutr*. 2011;105(5):755-764. doi:10.1017/S0007114510004319

86. Umbrello G, Esposito S. Microbiota and neurologic diseases: potential effects of probiotics. *J Transl Med*. 2016;14. doi:10.1186/s12967-016-1058-7

87. Thyroid Function Tests. American Thyroid Association. https://www.thyroid.org/thyroid-function-tests/. Accessed November 30, 2018.

88. Barnes Maze. http://www.sylics.com/contract-research/behavioural-tests/cognitive-tests/barnes-maze/. Accessed November 30, 2018.

89. P A. The Barnes Maze Test. Maze Engineers. July 2014. https://mazeengineers.com/mazes-basics-barnes-maze-test/. Accessed November 30, 2018.

90. The Novel Object Recognition Test. The protocol of NORT | Open-i. https://openi.nlm.nih.gov/detailedresult.php?img=PMC4280055_medscimonit-20-2733-g002&req=4. Accessed November 30, 2018.