Role of Gut Microbiota in the Production and Regulation of Serotonin Biosynthesis in the Gastrointestinal Tract

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Abstract

The gut microbiota, a complex ecosystem of microorganisms residing in the gastrointestinal tract, involved in the regulation of numerous physiological processes, including neurotransmitter synthesis. Among these, serotonin (5-hydroxytryptamine; 5-HT) a key neuromodulator has gained significant attention due to its dual regulation of both the enteric and central nervous systems. In the gastrointestinal tract, approximately 90% of the body's serotonin is synthesized by enterochromaffin cells, with gut bacteria significantly influencing this process through microbial metabolism of amino acid L- tryptophan. This thesis aims to investigate the role of gut microbiota in serotonin synthesis, focusing on its mechanistic contributions and regulatory pathways. A key regulatory mechanism involves the rate-limiting enzyme tryptophan hydroxylase (TPH), which facilitates the conversion of tryptophan into 5-hydroxytryptophan (5-HTP), a crucial serotonin precursor. To explore gut microbial contributions, eighty-two bacterial isolates and reference strains were screened for TPH activity, with nine strains further assessed for 5-HTP synthesis. Two strains were then identified and evaluated for their capacity to produce 5-HT, induce host cell responses, and exhibit probiotic properties. Our findings demonstrated that the gut microbiome exerts a diverse influence on serotonin synthesis, with certain microbial communities acting as more potent producers of 5-HTP than others, highlighting their potential role in serotonergic regulation and therapeutic applications.

Keywords: gut microbiota, serotonin synthesis, enterochromaffin cells, tryptophan hydroxylase, gut-brain axis, microbial metabolites.

Résumé

Le microbiote intestinal, un écosystème complexe de micro-organismes résidant dans le tube digestif, est impliqué dans la régulation de nombreux processus physiologiques, dont la synthèse des neurotransmetteurs. Parmi ceux-ci, la sérotonine (5-hydroxytryptamine; 5-HT), un neuromodulateur clé, a suscité un intérêt considérable en raison de sa double régulation des systèmes nerveux entérique et central. Dans le tube digestif, environ 90 % de la sérotonine de l'organisme est synthétisée par les cellules entérochromaffines, les bactéries intestinales influençant significativement ce processus par le métabolisme microbien de l'acide aminé L-tryptophane. Cette thèse vise à étudier le rôle du microbiote intestinal dans la synthèse de la sérotonine, en se concentrant sur ses contributions mécanistiques et ses voies de régulation. Un mécanisme de régulation clé implique l'enzyme limitante tryptophane hydroxylase (TPH), qui facilite la conversion du tryptophane en 5-hydroxytryptophane (5-HTP), un précurseur essentiel de la sérotonine. Afin d'explorer les contributions du microbiome intestinal, quatre-vingt-deux isolats bactériens et souches de référence ont été criblés pour l'activité TPH, et neuf souches ont été évaluées pour la synthèse de 5-HTP. Deux souches ont ensuite été identifiées et évaluées pour leur capacité à produire de la 5-HT, à induire des réponses cellulaires et à présenter des propriétés probiotiques. Nos résultats ont démontré que le microbiome intestinal exerce une influence diverse sur la synthèse de sérotonine, certaines communautés microbiennes étant plus productrices de 5-HTP que d'autres, soulignant leur rôle potentiel dans la régulation sérotoninergique et les applications thérapeutiques.

Mots-clés: microbiote intestinal, synthèse de sérotonine, cellules entérochromaffines, tryptophane hydroxylase, axe intestin-cerveau, métabolites microbiens.

Dedication

My deepest gratitude to God Almighty for all the opportunities that have come my way.

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As interest in the gut microbiome continues to flourish, the concept of harnessing serotonin biosynthesis and regulation as a probiotic trait presents a promising target for developing novel therapeutic treatments. Microbial communities in the mammalian gastrointestinal (GI) tract, function symbiotically with its host, influencing health and behavior [1–3]. Microbiota consists mainly of anaerobic bacteria, alongside archaea, viruses, fungi, and protozoa, and is shaped by various intrinsic and external factors [4]. This "metaorganism" functions in metabolism, immunity, and regulation of nervous system producing compounds, such as neurotransmitters, short-chain fatty acids, and various metabolites that communicate with the host through multiple pathways involving the vagus nerve, immune signaling, and direct bloodstream transport [5–7]. Its influence extends to endocrine regulation and has been linked to conditions including autism spectrum disorders, anxiety, depression, and Parkinson's disease [7]. Since identification of serotonin in the early 1900s and the recognition of GI tract, particularly enterochromaffin cells as a primary site of production, research has increasingly focused on the role of gut microbiota in serotonin synthesis and regulation. The gut microbiota influences serotonergic system through various routes including tryptophan metabolism, alters serotonin levels, and modulates serotonin receptors [7,8]. Albeit the physiological roles of serotonin and its intermediate molecule 5-hydroxytryptophan in bacteria remain partially understood. Evidence suggests that some bacteria may synthesize serotonin through mechanisms involving phenylalanine hydroxylase, while other studies propose its synthesis via tryptophan hydroxylase, with limited evidence supporting partial serotonin

production pathways [9,10]. Additionally, certain bacterial species produce related indole derivatives, that can impact host tryptophan availability [11]. In the last decade substantial progress has been made in identifying bacterial genes, such as aromatic amino acid hydroxylases (AAAH) and aromatic amino acid decarboxylases (AAAD) that are involved in serotonin biosynthesis pathway, however, the process of bacterial serotonin biosynthesis remains poorly understood [10]. Further research is required to confirm bacterial capabilities for serotonin synthesis and its potential implications for microbial and host interactions. By advancing our understanding of bacterial contributions to serotonin biosynthesis, this study aims to uncover novel probiotic candidates with potential therapeutic applications for modulating serotonergic function and gut-brain interactions.

CHAPTER 1:

Literature Review

Serotonin and the Gut Microbiome: Pathways, Functions, and Health Implications

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Abstract

Serotonin, an evolutionarily conserved molecule produced in the gut, plays a multifaceted role in host physiology. Several organs within the host can directly synthesize serotonin, yet its activity and secretion are intricately linked to the gut microbiome through both direct and indirect pathways. The gut microbiome significantly influences serotonin biosynthesis across the lifespan, with fluctuations reflecting parallel changes in serotonin levels. Dysbiosis of gut microbiota is associated with serotonergic dysfunction, contributing to various health disorders. Prebiotics and probiotics have shown the potential to modulate these effects and ameliorate related health issues. The interactions between the gut microbiome and serotonin metabolism underscore the importance of maintaining healthy microbiota for optimal serotonin function and overall health. This review explores the pathways through which the gut microbiome influences serotonin, its functions, and its implications for health, highlighting the therapeutic potential of targeting the gut microbiota to regulate serotonin levels.

Keywords: Serotonin, gut microbiome, tryptophan metabolism, gut-brain axis.

1. Introduction

Serotonin (5-hydroxytryptamine; 5-HT) is an evolutionarily conserved biogenic monoamine found in various species, from nematodes to humans. It was first isolated in 1937 from enterochromaffin (EC) cells in the intestine, where it is believed to induce muscle contraction in the gastrointestinal (GI) tract. Initially termed "enteramine," it was recognized as the primary secretory product of EC cells [12–14]. Nearly a decade later, researchers identified a compound in bovine serum capable of inducing blood vessel contraction, which they subsequently identified as serotonin [15,16]. Further molecular analysis confirmed that enteramine and serotonin are, in fact, the same molecule. In 1953, Betty Twarog made a significant breakthrough denoting its prominent role within the central nervous system, not just as a hormone but also as a neurotransmitter [17,18].

Since these foundational discoveries, serotonin has become a subject of considerable research due to its multifaceted role and involvement in the homeostatic regulation of both the central and enteric nervous systems [19,20]. Among the many classes of neurotransmitters, it has been established that 5-HT is highly expressed in the GI tract and accounts for the majority of 5-HT synthesized in the host [21]. Given that the intestine hosts a substantial population of microbiota, the interaction between intestinal microbes and host serotonin synthesis has been studied extensively [21,22]. It is estimated that gut microbes regulate approximately 50% of the 5-HT secreted in the intestine and are crucial for host tryptophan regulation, a 5-HT precursor [23]. Studies have reported that germ-free mice exhibit abnormally high concentrations of circulating tryptophan, suggesting a key role of gut microbiota in normalizing plasma tryptophan levels [24,25]. Additionally, germ-free mice express dysregulated 5-HT serum levels along with altered metabolite concentrations and changes in the expression of genes involved in 5-HT metabolism [23,26]. In contrast, conventional mice with typical gut microbiota have plasma 5-HT levels that are threefold higher than those of their germ-free counterparts [24]. Even though the majority of

dietary tryptophan is metabolized by the host via kynurenine pathway, approximately only 4-6% of dietary tryptophan is utilized by the intestinal microbiota [27]. Recent systematic review has shown that the gut microbiota have the capacity to modulate kynurenine metabolic pathway, particularly through inhibition of kynurenine rate-limiting enzyme, thereby reducing kynurenine metabolism and elevating intestinal serotonin synthesis [28,29]. Valladares et al. (2013) observed that administration of the commensal bacterium *Lactobacillus johnsonii* inhibited the activity of kynurenine rate-limiting enzyme indoleamine 2,3-Dioxygenase, reduced serum kynurenine and elevated 5-HT levels [30]. Although the serotonin biosynthetic pathway is well understood in eukaryotes, it remains largely unexplored in bacteria. It is unclear whether the gut microbiota strictly influence serotonin synthesis by regulating EC cells or independently facilitate *de novo* synthesis of 5-HT and its precursors. Tracing changes in gut microbial composition at different stages of life has revealed an association with host homeostasis and neuro-immune interactions [31]. Thus, establishing the connection between the aging microbiome and its effects on serotonin synthesis is essential for gaining better insight into the intricate influence of gut microbiota on the serotonin biosynthetic pathway and its broader impact on physiological and neurological functions. Despite growing interest in this field, many questions remain regarding the mechanism by which intestinal gut microbiota influence serotonin synthesis and the associated effects of the changing microbiome throughout the lifespan. To date, the microbial contribution to host serotonin levels is primarily understood to occur through the modulation of the host's serotonin production and metabolism. However, some promising studies have suggested direct microbial synthesis of serotonin [8,32]. For example, *Pseudomonas putida* KT2440 possesses aromatic L-amino acid decarboxylase (AAAD) genes capable of converting 5-HTP to serotonin, while *Pseudomonas fluorescens* RG11 was shown to produce serotonin via phenylalanine hydroxylase (PheH), which

hydroxylates tryptophan to 5-hydroxytryptophan (5-HTP) despite its primary role in phenylalanine metabolism. The authors also showed that mutant strains lacking functional PheH exhibited significantly reduced serotonin production [33,34].

The multiple steps involved in serotonin biosynthesis add additional complexity to our understanding of the role of the gut microbiota, as it can be regulated through multiple pathways. Research has shown that intrinsic and extrinsic factors, including age, dietary intake, and exercise, significantly influence microbiome development and overall well-being, directly impacting serotonin metabolism [35]. For instance, voluntary exercise has been found to restore gut microbial composition, leading to downstream effects on host 5-HT levels [36]. A shift toward a more favourable microbial composition strengthens the serotonergic system, similar to the effects observed with age- and diet-related changes, which influence metabolite secretion closely tied to 5-HT regulation in the host [37–41]. Understanding the extent to which the gut microbiome influences serotonin metabolism is crucial as it has significant implications for host health. This review aimed to examine the extent to which the gut microbiome influences serotonin biosynthesis and its various direct and indirect pathways that modulate 5-HT synthesis, shedding light on its potential therapeutic applications for the treatment of various diseases related to serotonergic dysfunction. The literature search was conducted using databases such as Google Scholar, PubMed, and Scopus with keywords including "serotonin," "gut microbiome," "tryptophan metabolism," and "gut-brain axis." Studies published in English between 2000 and 2024 were considered. Inclusion criteria focused on peer-reviewed articles that explored the interaction between gut microbiota and serotonin pathways; studies were excluded if they lacked relevance to microbiome-mediated serotonin biosynthesis or were not original research.

2. Basis of the Serotonergic System in the GI tract

The serotonergic system is often regarded as a singular entity; however, it is composed of several subsystems found in many organs with similar biosynthetic and degradation pathways, along with the modulation of a common set of receptors [42]. Serotonin synthesis begins with the metabolism of L-tryptophan to 5-HTP by tryptophan hydroxylase (TPH), which exists in two isoforms: TPH1 in peripheral cells and TPH2 primarily in the central nervous system except some peripheral cells, such as myenteric neurons, also utilize TPH2. This is followed by decarboxylation of 5-HTP to 5-HT by aromatic L-amino acid decarboxylase (AADC) [43,44]. In the central nervous system, 5-HT neuronal cells originate from the hindbrain, mainly clustering in the raphe nuclei within the brainstem, producing approximately 5% of the body's serotonin designated as B1-B9 [42,45]. Conversely, in the periphery, the majority of 5-HT is synthesized in EC cells, some is also produced in mast cells, myenteric neurons, mammary epithelial cells, melanocytes, fibroblasts, osteoclasts, pinealocytes, pancreatic beta cells, pulmonary artery endothelial cells, keratinocytes, adipocytes, and hepatocytes [46].

The effects of serotonin are largely governed by its interaction with receptors on the plasma membrane. To date, 14 distinct serotonin receptor (5-HTR) subtypes have been identified in humans, grouped into seven families, HTR1-HTR7, according to their corresponding structural, pharmacological, and signal transduction properties, all of which are G protein-coupled receptors that initiate intracellular signaling cascades, with the exception of the ligand-gated cation channel HTR3 [46,47]. Another receptor independent mechanism is serotonylation, a post-translational modification in which serotonin exerts a long-lasting intracellular effect by covalently linking glutamine residues in proteins through transglutaminases [48]. Due to its hydrophilic nature and positive charge at physiological pH, 5-HT requires specialized transmembrane proteins to bypass the cellular plasma membrane. These proteins, which play a key role in terminating receptor-

mediated serotonin signaling, fall into two categories with distinct kinetic properties [46,49]. Specialized transmembrane proteins categorized into the high affinity/low capacity (uptake-1) system constitute a serotonin-selective reuptake transporter (SERT) expressed in the axonal terminals of serotonergic neurons, platelets, and broadly distributed in the periphery. The second category is the low-affinity/high-capacity (uptake-2) system, which consists of organic cation transporters (OCTs 1, 2, and 3) and the plasma membrane monoamine transporter (PMAT). Unlike SERT, this system is less selective, transporting multiple monoamines, in addition to serotonin. Despite its hydrophilic nature, substantial evidence suggests that 5-HT can cross cell membranes via a diffusion-like process [46,50–53]. At serotonergic nerve terminals, monoamine oxidase A (MAO-A) degrades serotonin and converts it into 5-hydroxyindole acetic acid (5-HIAA) using aldehyde dehydrogenase [54].

In the GI tract, EC cells only make up around ~1% of the total enteric epithelial cells in humans, which are subtypes of enteroendocrine cells found among enterocytes in the intestinal epithelium responsible for synthesizing, storing, and releasing serotonin, accounting for 90% of circulating serotonin in the body and 95% of serotonin released in the gut, whereas enteric neurons and other cell types produce the remaining 5% [55,56]. In EC cells, TPH1 initiates serotonin synthesis by hydroxylating tryptophan intracellularly. The synthesized 5-HT is then packaged and stored in vesicles via vesicular monoamine transporter-1 (VMAT-1) only present in neuroendocrine cells, while VMAT-2 is found in neuronal cells [56,57]. Serotonin vesicles are primarily stored at the basal border or apical membrane and released in response to mechanical and chemical stimuli, including changes in pH, luminal pressure, and chemical stimulation such as nutrients, toxins, and endogenous chemical stimuli [58,59]. Upon vesicle release, it follows several pathways: it is either released into the intestinal lumen or traverse to the lamina propria, where it interacts with epithelial

cells, nerve terminals, and immune cells by acting on multiple 5-HTRs [55,59]. Notably, serotonin is released in a paracrine manner, where its concentration determines receptor activation: higher levels stimulate intrinsic or extrinsic sensory nerve terminals through 5-HTR3, whereas lower levels stimulate 5-HTR1P or 5-HTR4 [56]. Since 5-HT cannot easily penetrate the lipid plasma membrane, transporter-mediated uptake is required to prevent excessive activation or receptor desensitization. The activity of 5-HT in the GI tract is terminated by the uptake of epithelial cells via SERT and degradation to 5-HIAA by MAO_A [55,60]. Additionally, serotonin released in the lamina propria may enter portal circulation, where it can be present in a free form or taken up by platelets via SERT. Before entering systemic circulation, the portal circulation must be processed by the liver, where one-third of the free serotonin catabolized rapidly into 5-HIAA by MAOA, whereas the remaining two-thirds of 5-HT is degraded to 5-HT-*O*-glucuronide by glucuronidases. Nevertheless, only platelet stored 5-HT is protected from degradation and can enter circulation [56].

The GI tract is extensively innervated by a dense network of neurons that travels through the vagus nerve, forming the enteric nervous system. In the presence of dietary substances, signals are transmitted to the central nervous system such as the magnus and nucleus solitarius via the vagus nerve. Serotonergic neurons in the brainstem, particularly the raphe nuclei, influence GI tract function via descending pathways that modulate the enteric nervous system, inducing the release of 5-HT from EC cells and subsequently initiating peristalsis and digestion [61,62]. To maintain appropriate 5-HT concentration in the GI tract, the serotonergic system relies on a complex feedback mechanism. Positive feedback is sustained through efferent signals via the vagus nerve, while negative feedback loops primarily operate through humoral signals, including insulin, leptin, glucose, and ghrelin, traversing the circulatory system to the arcuate nucleus of the hypothalamus,

activating proopiomelanocortin neurons. Subsequently, stimulating the release of alpha-melanocyte stimulating hormone, which functions as an inhibitory neuromodulator of the raphe nuclei and nucleus solitarius, halts the descending signals that mediate peristalsis in the GI tract [62,63]. The presence of negative feedback is crucial, as elevated concentrations of 5-HT can be detrimental to the host, increasing intestinal motility and potentially leading to nausea, diarrhea, flushing, and heart palpitations, which are associated with intestinal disorders, carcinoid syndrome, cholera, chemotherapy, and radiation therapies [64].

3. Serotonin: a multifunctional neurotransmitter

3.1. Neuronal Signaling

5-HT has been recognized since its discovery for its widespread role in the host. The central and peripheral serotonergic systems control numerous physiological and behavioral processes [65]. Achieved by serotonergic neurotransmission through different receptor subtypes, in addition to receptor-independent signaling that may induce long-lasting effects, researchers believe that it may influence cognition, emotion, and the generation of dendritic spines and neuronal cells by perpetuating gene expression patterns [48,66]. Certainly, central serotonergic neurotransmission governs emotional memory, recognition of facial emotions, attentional bias, decision-making, and dysfunctional attitudes [67]. Studies have postulated that negative biases in information processing contribute to the persistence of major depressive disorder [68]. In some areas of the limbic system, serotonergic signals are transmitted in a paracrine fashion [67], and mood/emotion is associated with 5-HT synaptic availability, underpinning the action of several antidepressants. 5-HT can also act as an autocrine neurotransmitter, potentiating melatonin secretion from the pineal gland [69]. This explains the significant role of 5-HT within the CNS in sleep, learning, memory, pain perception, reward circuitry, and appetite regulation [65,70]. Studies have found that stimulation of the hypothalamus's arcuate nucleus induces anorectic responses, as serotonin inhibits orexigenic

peptides and stimulates anorexigenic peptides when it receives peripheral signals, suppressing appetite, whereas serotonin depletion results in weight gain and hyperphagia [71,72]. Similarly, in the periphery, intrinsic and extrinsic afferent neurons are stimulated in a paracrine manner, modulating secretory and peristaltic reflexes and maintaining GI tract homeostasis by controlling the secretion of fluids and electrolytes in the intestine [49,73–75]. Perturbation of the serotonergic system in the GI tract has been implicated in several GI disorders, including irritable bowel syndrome, inflammatory bowel disease, celiac disease, and colorectal cancer [76].

3.2. Metabolic and Hormonal Signaling

The availability of 5-HT in peripheral tissues is influenced by both local production from pancreatic β cells, adipocytes, and osteoclasts, and by the concentration of free circulating 5-HT in the bloodstream that can function directly as a hormone [77]. Insulin plays a central role in maintaining nutrient homeostasis, and the interplay between insulin and serotonin is crucial for coordinating metabolic processes and maintaining the overall physiological balance [78–81]. Serotonin regulates insulin secretion through both receptor dependent signaling and receptor independent mechanisms discussed by Bascuñan et al. (2018) [80]. Paulmann et al. (2009) observed that insulin secretion is positively correlated with the intracellular concentration of 5-HT, which also possesses the capacity to suppress insulin secretion [82]. Impairment of insulin secretion, as seen in *Tph1*-/- deficient mice, causes glucose intolerance and the development of diabetes [82]. During periods of high metabolic demands, such as pregnancy, 5-HT concentration is elevated, which in turn promotes β cells adaptive proliferation and insulin secretion [83]. The liver, primarily influenced by insulin and glucagon, is an important regulator of blood glucose and lipid levels. Evidence shows that peripheral 5-HT directly affects hepatocytes, promoting gluconeogenesis and glycolysis while reducing triglyceride and cholesterol levels [77]. Adipose tissues serves as the main energy reservoirs for storing nutrients and secreting various adipokines

to maintain systemic energy homeostasis [84]. Peripheral serotonin affects adipocyte function by promoting lipolysis in white adipocytes via the 5-HTR2b, which activates hormone-sensitive lipase, impairs insulin action and glucose uptake [77]. Serotonin stimulates adipocyte differentiation and influences lipid metabolism via various receptors and autocrine mechanisms. In brown and beige adipocytes, it mainly acts on the 5-HTR3a, inhibiting thermogenesis by blocking β-adrenergic signaling and reducing uncoupling protein-1 expression, leading to decreased energy dissipation [77]. Peripheral serotonylation also has a significant impact on insulin release, muscle contraction, platelet aggregation, and pulmonary hypertension [66]. Furthermore, there is a clear association between serotonin and growth factors, which has been postulated to enhance the regional expression of growth factors and induce the activation of the ERK and Akt pathways through 5-HTRs, thereby promoting neurogenesis and plasticity [85].

3.3. Immune Signaling

The serotoninergic system regulates both the innate and adaptive immune systems. Granted that some immune cells, such as T cells, monocytes/macrophages, and mast cells, produce 5-HT and may encounter 5-HT in the lamina propria, platelets are considered the primary source of 5-HT for immune cells and lymphoid tissues [86]. Upon the activation and release of 5-HT from platelets, the innate immune system is stimulated as the body's first line of defense against pathogens. This response is characterized by rapid and non-specific actions attributed to the 5-HTRs present in immune cells [86]. Moreover, components of serotonergic machinery including SERT, MAOA, TPH, and 5-HTRs have been detected in several innate immune cells contributing to regulatory effects such as, anti-inflammatory responses, cell polarization, migration, recruitment, and the release of chemokines and cytokines [87,88]. 5-HT can also stimulates proinflammatory cytokines and natural killer cells, enhances cytotoxicity, and exerts antiviral activity. Increasing serotonin levels through selective serotonin reuptake inhibitors (SSRIs) resulted in higher numbers of natural

killer cells [88–90]. Conversely, serotonin is crucial in adaptive immunity, in which the defense is selective and responsible for immunological memory. Its specificity arises from the antigen-specific recognition by T and B lymphocytes. T cells express TPH, SERT, MAOA, and various 5-HTRs. B cells while not known to express TPH or MAOA, multiple serotonin receptors, including 5-HTR1A, 5-HTR1B, 5-HTR7 and 5-HTR3A have been detected. Activation of 5-HTR1A promotes proliferation via activation of nuclear factor kappa beta (NF-κB) pathway, whereas blocking 5-HTR1A decreases B cell lymphoma growth. Although SERT expression in normal B cells remains less clear, it is present in malignant B cells, suggesting altered serotonin handling in pathological states. Therefore, serotonin can directly influence both T and B lymphocytes via their respective 5-HTRs or stimulate antigen-presenting cells, such as dendritic cells and macrophages, which then induce adaptive immune responses [86–88,91].

4. Serotonin biosynthesis pathways

4.1. Host Metabolism

The initial crucial step in the serotonin biosynthesis pathway involves the utilization of tryptophan (**Figure 1.1**), mainly obtained through the diet, by a member of a family of pterin-dependent aromatic amino acid hydroxylases – TPH [11,92,93]. Dietary proteins digested in the small intestine release tryptophan, which traverse through the bloodstream following intestinal absorption [11]. While mammals cannot directly synthesize tryptophan, studies have shown that it can be readily synthesized by some intestinal microbial residents. However, it is unknown whether microbial tryptophan is utilized by the host [94]. Approximately 10-20% of tryptophan found in plasma is in a free form, and circulating tryptophan is commonly bound to albumin, expressing diurnal and seasonal variations, and impacted by age, sex, dietary changes, and drug intake. It has not been elucidated whether albumin can regulate tryptophan availability for tissue metabolism [93,95–98], even though tryptophan dissociation from albumin induces a sustained elevation in 5-

HT synthesis, as the rate of its synthesis depends on the concentration of free circulating tryptophan [99]. Free fatty acids are also involved in the regulation of this mechanism. The sympathetic nervous system controls the concentration of circulating free fatty acids, and under acute stress and physical exercise, the sympathetic response increases the levels of free fatty acids, promoting their binding to albumin. This reduces tryptophan's affinity to albumin and elevates free tryptophan levels in plasma [100]. Most of the total tryptophan absorbed goes through oxidation in the liver via the kynurenine pathway, by which tryptophan availability is mainly regulated. Tryptophan can also be catabolized by L-amino acid oxidase resulting in indole-3-pyruvate production (**Figure 1.1**) [101]. Since 5-HT synthesis relies on the presence of tryptophan, 5-HT synthesis is dose-dependent on tryptophan availability [102,103], and only 3% of dietary tryptophan is utilized for serotonin synthesis [104]. An increase in kynurenine metabolism can alter serotonin levels by depriving the TPH of its substrate, as seen in major depressive disorders. Elevated proinflammatory cytokines further induce the activation of the indoleamine 2,3-dioxygenase enzyme, favoring kynurenine metabolism, thereby reducing tryptophan for the 5-HT metabolic pathway. Individuals with major depressive disorder have been shown to have higher levels of inflammatory cytokines in the cerebrospinal fluid and peripheral blood, as well as an increase in kynurenine, suggesting that a shift in kynurenine and serotonin metabolic pathways affects 5-HT synthesis in the host, which is supported by the monoamine hypothesis [100,105–110]. Several cofactors are involved in initiating the breakdown of tryptophan and subsequent metabolism of 5-HT. For instance, the activity of TPH monooxygenase enzyme is highly regulated by the cytosolic concentration of tetrahydrobiopterin (BH4) cofactor (**Figure 1.1**). BH4 is found in most mammalian cells and studies indicate that the intestinal microbiota may also produce BH4 [111,112]. Inflammatory cytokines can also alter cofactor activity through multiple mechanisms,

including generation of an oxidative state, causing irreversible degradation of BH4 [113]. Additionally, factors such as iron and vitamins availability can impact TPH activity (**Figure 1.1**). Iron, in particular, acts as an obligatory cofactor by enabling TPH catalytic activity, and its deficiency has been linked to impaired serotonin synthesis as well as reduction in short-chain fatty acids (SCFAs) and SCFAs producing gut microbiota [114,115]. These disruptions contribute to lower serotonin levels and have been associated with mood disorders [116,117]. Similarly, vitamins B9 and B12 are involved in S-adenosyl-l-methionine synthesis, a naturally occurring molecule that serves as a methyl-donating cofactor in the rate-limiting step. Deficiencies in both B9 and B12 are associated with cognitive deficits, depression, and a low response to antidepressant treatments, potentially due to reduced S-adenosyl-l-methionine levels. While using S-adenosyl-l-methionine as an adjunct to antidepressants seems to confer a greater response, its use as the sole antidepressant agent has also been reported [118–120]. Gene analysis of individuals with IBS and control subjects revealed that the serotonergic pathway was the most distinctively expressed gene set. Lower *TPH1* expression was positively correlated with vitamin D serum concentration, and the treatment of intestinal cells with vitamin D ameliorated the downregulation observed in individuals with IBS [121]. Grozic et al. (2022) further confirmed the genetic differences related to the serotonergic pathway as well as a positive correlation between vitamin D and serotonin synthesis in the periphery [122]. However, Paterick et al. (2014) found that vitamin D upregulated *TPH2* expression while downregulating *TPH1* transcription in the periphery [123]. Furthermore, the decarboxylation of 5-HTP into 5-HT depends on vitamin B6 bioavailability [93], a deficiency of which impairs tryptophan metabolism and decreases serotonin levels [11,124,125] by reducing AADC activity [126]. There is ample evidence that indicates that deficiency and low intake of vitamin B6 is associated with cognitive impairment, depression, anxiety, inflammatory bowel

disease (IBD), irritable bowel syndrome (IBS), and colorectal cancer, all of which are affected by alterations in serotonin levels [127–137]. One of the core etiologies prevalent in autism spectrum disorder is the impairment of the serotonergic system and reduction in serotonin synthesis. Studies have demonstrated that vitamin B6 supplements are effective in alleviating some of the symptoms experienced by individuals with autism [138], and in mouse models, fecal microbial transplantation alleviated autism-like behavior and restored vitamin B6 homeostasis [139].

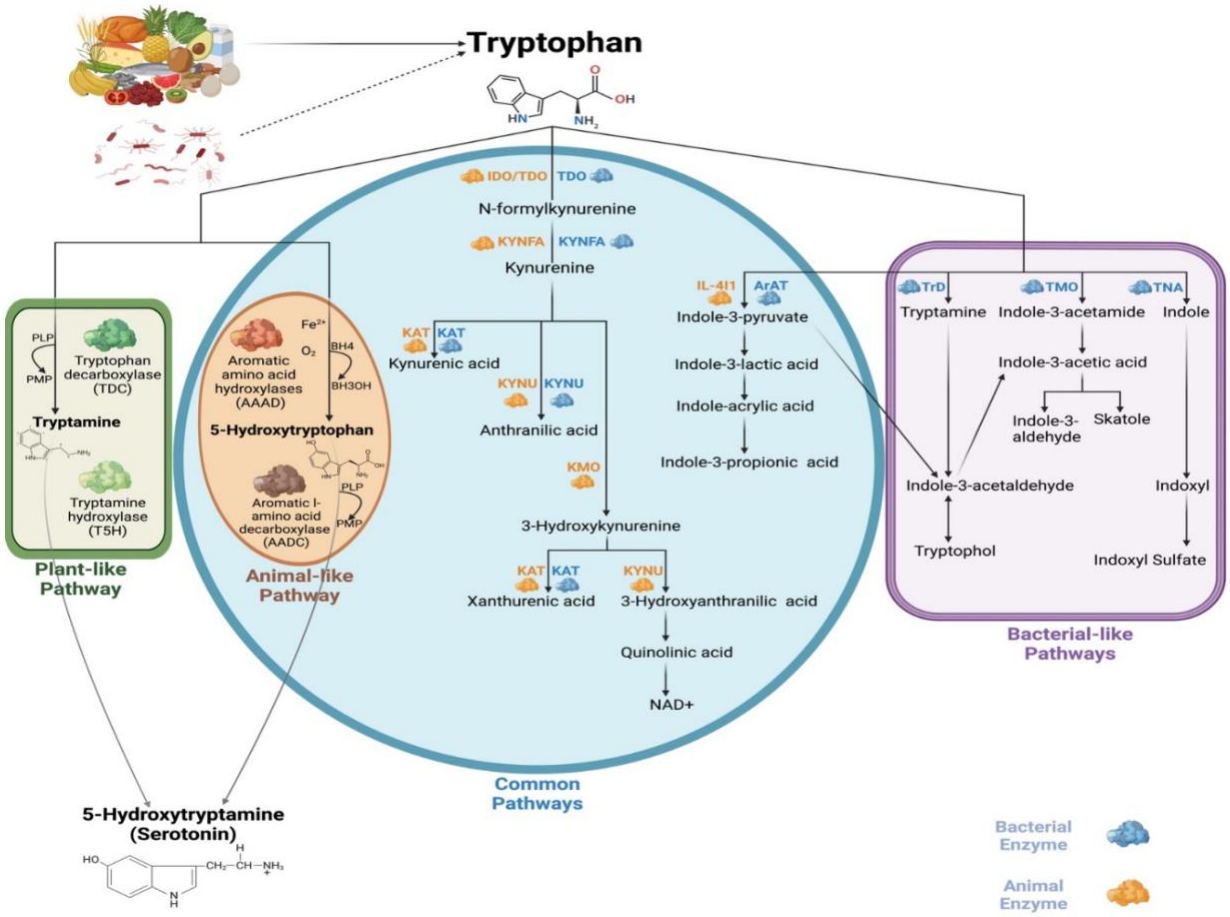

Figure 1.1. Overview of three major tryptophan metabolic pathways pertaining to serotonin, kynurenine, and indole pathways through plant like pathway (green frame), animal pathway (brown frame), common pathways between animal and bacteria (Blue frame) and bacterial like pathways (purple frame). Abbreviations: PLP, Pyridoxal 5′-phosphate; PMP, Pyridoxamine phosphate; Fe^{2+}, Iron(2+); O_2, Molecular oxygen; BH4, Tetrahydrobiopterin; BH3OH, 4a-hydroxytetrahydrobiopterin; IDO, indoleamine 2,3-dioxygenase; TDO, tryptophan 2,3-dioxygenase; KYFNA, Kynurenine formamidase; KAT, kynurenine aminotransferase; KYNU, kynunerinase; KMO, kynurenine 3-mono-oxygenase; NAD+, nicotinamide adenine dinucleotide; IL-4I1, interleukin-4-induced-1; ArAt, aromatic amino acid transaminase; TMO, tryptophan-2-monooxygenase; TNA, tryptophanase; TrD, tryptophan decarboxylase.

4.2. Role of Gut Microbiome in 5-HT biosynthesis

4.2.1. Direct pathways

Several bacterial species residing in the intestine possess the genetic capacity to synthesize tryptophan via *de novo* biosynthetic pathways [140]. One such pathway, known as the shikimate pathway, involves a series of enzymatic reactions that culminate in the conversion of a common aromatic amino acid precursor, chorismate, into tryptophan (**Figure 1.2A**) [141,142]. Although most tryptophan amino acids obtained through diet are ingested and metabolized by the host, some tryptophan can still travel to the large intestine and be utilized by gut microbes to generate several metabolites and limit tryptophan availability to the host [11,143]. Germ-free mice have higher plasma tryptophan levels than conventional mice [144]. Meanwhile, depletion of host tryptophan is associated with a reduction in microbial proliferation, and the use of antibiotics diminishes tryptophan availability in the jejunum along with a reduction in microbial tryptophan decarboxylation activity in the colon [11]. Earlier studies have shown that bacterial flora isolated from the ascarid *Ascaris suum* (a large ascarid nematode) were able to produce significant amounts of 5-HT in the culture medium, independent of tryptophan supplementation [145,146]. It is widely accepted that EC cells are the chief producers of 5-HT in the periphery, and intestinal bacteria merely act as inducers or regulators. However, recent landmark studies have demonstrated that certain bacterial species can synthesize serotonin (**Figure 1.2A**). Sanidad et al. (2024) found that gut bacteria isolated from neonatal mice and healthy human infants directly secrete 5-HT, predominantly by *Enterococcus gallinarum* and *Klebsiella grimontii*, respectively [147]. Similarly, probiotic *Lactobacillus* species have been shown to secrete 5-HT and its metabolite, 5-HIAA (**Figure 1.2A**) [148]. *De novo* synthesis of 5-HT has also been detected in yeast from the genera *Pichia* and *Saccharomyces* [32,149]. Additionally, TPH1 enzyme activity and 5-HTP synthesis were detected in intestinal microbiota, along with the synthesis of several rate-limiting co-factors,

including BH4 and S-adenosyl-l-methionine, and the presence of AADC enzyme was found in *Enterococcus, Blautia, Clostridium, Ruminococcus, and Tyzzerella* (**Figure 1.2A**) [9,120,150,151]. Luqman et al. (2018) observed that staphylococci harbored in the human gut utilizes staphylococcal aromatic amino acid decarboxylase (SadA) for serotonin synthesis by decarboxylating 5-HTP to 5-HT [152]. Moreover, phenylalanine hydroxylase exhibits a hydroxylation activity similar to that of TPH1. Some bacterial species carry genes encoding phenylalanine hydroxylase, indicating that intestinal bacteria may hydroxylate tryptophan through multiple aromatic amino acid hydroxylase enzymes (**Figure 1.2A**) [153,154]. Despite the inherent persistence of enzyme promiscuity, and the serotonin pathway in the intestine may resemble that of the host, an alternative pathway analogous to plant synthesis has been proposed (**Figure 1.1**) [153]. For instance, inhibition of microbial TPH activity using n-chlorophenylalanine has been shown to affect the growth of *Candida guillermondii*, whereas it does not seem to confer any effects on *Escherichia coli* [155]. Williams et al. (2014) reported that gut microbiota such as *Clostridium sporogenes*, *Ruminococcus gnavus*, and Bacillota decarboxylate tryptophan into tryptamine. Subsequent analysis of the Human Microbiome Project data revealed that at least 10% of the human population harbors tryptophan decarboxylase enzymes. However, it has not been elucidated whether tryptamine is converted to serotonin in bacteria or whether it induces serotonin synthesis in EC cells (**Figure 1.2 A,C**) [156]. Additionally, a recent metagenomic analysis revealed that 20% of gut-associated integrated microbial genomes were linked to the animal-like pathway, while plant-like pathway was relatively uncommon [157]. Similar to host metabolic processes, the intestinal microbiota is also likely to metabolize tryptophan via the kynurenine pathway through the action of four key enzymes and their associated genes. This suggests that gut microbiota have

the mechanistic capability to harness tryptophan across multiple metabolic pathways (**Figure 1.1**) [32,158–160].

4.2.2. Indirect pathways

4.2.2.1 Vitamins and Metabolites Secretion

In addition to the direct synthesis of 5-HT, gut bacteria play a significant role in modulating serotonin expression and release in the GI tract. Intestinal microbial residents have exceptional abilities to synthesize and utilize vitamin B6. Genomic analysis of 256 human-derived gut bacteria estimated that 40-65% of gut bacteria predominantly belonging to the *Bacteroides* genus, possess pathways for vitamin B synthesis, out of which only four B vitamins, including B3, B6, B9, and B12 secreted in adequate amounts and can be potentially utilized by the host (**Figure 1.2B**) [161,162]. Pyridoxal 5′-phosphate (PLP) is the active form of vitamin B6, and the PLP-dependent enzyme AADC has been detected in commensal gut bacteria, stimulating the production of tryptamine [163,164]. Gut microbial metabolites are increasingly recognized for their significant influence on host 5-HT synthesis, one potential mechanism could be through microbial synthesis of essential vitamins. It is believed that microbial secretion of vitamin B6 or PLP may influence EC cell activity by AADC, which depends on PLP availability (**Figure 1.2B**) [164]. While vitamin B6 is integral to tryptophan metabolism, tryptophan metabolites can also affect vitamin B6 metabolism [165]. The host 5-HT metabolic process also requires a sufficient supply of vitamins B9 and B12, and intestinal bacteria may influence host serotonin secretion by replenishing the host's need for these vitamins (**Figure 1.2B**) [29]. Moreover, bacterial fermentation by-products, such as SCFAs such as propionate, butyrate, and acetate, regulate serotonin biosynthesis via stimulation of EC cell serotonin secretion, upregulating colonic *TPH-1*, downregulating *MAO-A,* and activating 5-HTRs (**Figure 1.2C**) [166]. Approximately 5% of tryptophan is metabolized by intestinal bacteria into various indole derivatives (**Figure 1.1**), including tryptamine and indole-3-

acetic acid, which are associated with host serotonin synthesis (**Figure 1.2C**) [8,11,32,167]. Indole mutant mice display a reduction in colonic EC cells and serotonin serum levels, whereas indole supplementation amplifies the serotonin pathway [168,169]. Yano et al. (2015) reported that spore-forming bacteria isolated from SPF mice and healthy human colon produce metabolites, including tryptamine, α-tocopherol, and p-aminobenzoate, which induce colonic 5-HT synthesis *in vitro* and *in vivo* (**Figure 1.2C**) [117]. As mentioned, intestinal microbiota are capable of synthesizing 5-HTP; however, subsequent analysis by Zhang et al. indicated low levels of 5-HT in intestinal microbiota, suggesting that there may be an alternative pathway [9]. Recent study indicates that intestinal bacteria metabolize exogenous 5-HTP to 5-hydroxyindole through tryptophanase, inducing 5-HT secretion from intestinal cells (**Figure 1.2A**) [170].

4.2.2.2 Biotransformation

Peripheral catecholamines are often conjugated with either glucuronide or sulfate, which are biologically inactive forms. Mammalian hosts rely on commensal gut microbiota for the deconjugation process, as they possess β-glucuronidase activity [171,172]. Similarly, indolamines, such as 5-HT, released in the conjugated form in the GI tract require the presence of gut microbiota for the deconjugation of glucuronide-conjugated 5-HT. Indeed, germ-free mice express high levels of conjugated 5-HT, and subsequent recolonization with specific pathogen-free fecal microbiota induces the deconjugation of 5-HT, elevating the free 5-HT form (**Figure 1.2A**) [173]. Moreover, the gut microbiota also facilitates the dihydroxylation and deconjugation of primary bile acids into secondary bile acids, such as lithocholic acid and deoxycholic acid. These secondary bile acids increase colonic serotonin levels and *TPH-1* expression in intestinal cells (**Figure 1.2 A,C**) [117,174–177]. For instance, administration of *Bacillus subtilis* enhances bile acid metabolism, inducing the release of 5-HT from EC cells through activation of the TGR5/TRPA1 bile acid receptor pathway [178]. In addition, spore-forming bacteria are highly associated with serotonin

induction and have been shown to contain high 7α-dehydroxylation activity essential for secondary bile acid metabolism, thereby increasing deoxycholate concentration in the colon [117]. Dietary polyphenols are naturally found in their conjugated forms. As a result, approximately only 5-10% of dietary polyphenols are absorbed, whereas the majority of polyphenols are processed by the gut microbiota in the large intestine, where microbial enzymes engage in the deconjugation process (**Figure 1.2A**) [179]. Deconjugation of polyphenols releases bioactive compounds, which may facilitate 5-HT synthesis (**Figure 1.2C**). A previous study showed that *Lactiplantibacillus plantarum* and *Streptococcus thermophilus* metabolize mulberry anthocyanins into various bioactive compounds, including chlorogenic acid, cypto-chlorogenic acid, cyanidin-3-glucoside, ferulic acid, and caffeic acid [180,181]. Interestingly, chlorogenic acid was found to significantly elevate 5-HT serum levels and promote the diversity of gut microbiota associated with colonic 5-HT secretion (**Figure 1.2C**) [182], whereas anthocyanin aglycons, such as cyanidin-3-glucoside, were able to inhibit MAO-A and -B isoform activity, thereby modulating the serotonergic pathway (**Figure 1.2C**) [183,184].

4.2.2.3 Immunomodulation

The mammalian GI tract constitutes the largest part of the immune system, where the gut microbiota exerts a greater influence on immune function [185,186]. Emerging evidence suggests that the gut microbiota can indirectly regulate the host serotonergic system through modulation of immunological responses. The immune system utilizes pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs), to detect microbial molecules and initiate immune signaling cascades. Activation of these PRRs has been shown to regulate SERT activity, 5-HT receptor expression, and 5-HT signaling pathways (**Figure 1.2D**) [187,188]. Wang et al. (2019) observed that activation of TLR2 in EC cells upregulated *TPH1* expression and 5-HT secretion, whereas its deficiency correlated with a

reduction in EC cells and decreased 5-HT levels in the gut [189]. Meanwhile, other studies have reported that the activation of TLR3/TLR4 inhibits SERT activity and the activation of NOD1/NOD2 reduces SERT activity in intestinal epithelial cells [187]. In addition, analysis of intestinal tissue and peripheral blood revealed that intestinal bacteria can induce the activation of $CD4^+$ T cells, resulting in the release of secretory molecules that induce EC cells 5-HT secretion [190,191]. Furthermore, commensal intestinal bacteria and their indole derivatives modulate the activity of anti-inflammatory cytokines ameliorating inflammation and, in turn, affects the kynurenine/serotonin metabolic ratio. Studies have found that *Bifidobacterium longum* subsp. *infantis* and *Limosilactobacillus reuteri* produce indole-3-acetate, resulting in the attenuation of proinflammatory cytokine interleukin-8 responses along with promoting $CD4^+$ T-cell differentiation (**Figure 1.2D**) [192–194]. Microbial tryptophan-derived indole, tryptophan, and indole-3-carboxaldehyde activate the immunomodulating receptor transient receptor potential ankyrin A1 (Trpa1) in the intestines of humans, zebrafish, and mouse models. Activation of Trpa1 facilitates the release of 5-HT from intestinal enteroendocrine cells (**Figure 1.2D**) [195]. Similarly, treatment with the commensal bacterium *Faecalibacterium prausnitzii* inhibited proinflammatory cytokines both *in vitro* and *in vivo* (**Figure 1.2D**) [196]. Some bacterial strains have been found to stimulate the release of interleukin-12 to various degrees, which promotes the breakdown of 5-HT. Therefore, elevated interleukin-12 levels are associated with reduced 5-HT levels [197,198].

4.2.2.4 Comparative analysis of microbiome serotonin regulation

The modulation of serotonin pathways by gut microbes involves distinct mechanisms depending on whether microbial activity occurs extracellularly or intracellularly, although current research primarily focuses on extracellular interactions. Extracellular microbes, such as spore-forming *Clostridia* and *Akkermansia muciniphila*, influence serotonin production through metabolite

signaling and extracellular vesicles. These bacteria stimulate EC cells in the colon to synthesize serotonin by upregulating *Tph1*, a key enzyme in serotonin biosynthesis [117,199]. Microbial metabolites such as SCFAs directly enhance serotonin production in EC cells, as demonstrated in germ-free mice recolonized with spore-forming bacteria [117]. Additionally, bacterial extracellular vesicles from species such as *A. muciniphila* and *E. coli* modulate serotonin bioavailability by increasing SERT expression and reinforcing gut barrier function via aryl hydrocarbon receptor (AHR) activation [200,201]. Extracellular microbes also compete with the host for tryptophan, diverting it into the kynurenine pathway and reducing its availability for serotonin synthesis [199,202]. In contrast, intracellular microbial modulation of serotonin remains less characterized in the provided studies. While no direct evidence of intracellular serotonin synthesis by microbes is described, certain mechanisms are hypothesized. For example, microbial metabolites like SCFAs may penetrate host cells to influence intracellular signaling pathways, potentially affecting central serotonin levels in the brain [201,203]. Pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs), which are located on or within host cells, might mediate interactions between microbial components (e.g., microbial-associated molecular patterns, MAMPs) and the serotonergic system [201]. However, the current literature emphasizes extracellular mechanisms, with limited data on how intracellular microbes-or their components-directly regulate serotonin pathways. The interplay between these modulatory roles highlights the microbiota's capacity to influence both peripheral and central serotonin systems. Extracellular microbes predominantly act via environmental signaling, while intracellular effects may involve secondary metabolite diffusion or host cell receptor interactions [201,204]. Further research is needed to clarify the contributions of intracellular related microbial activity to serotonin regulation, particularly in the context of gut-brain axis communication.

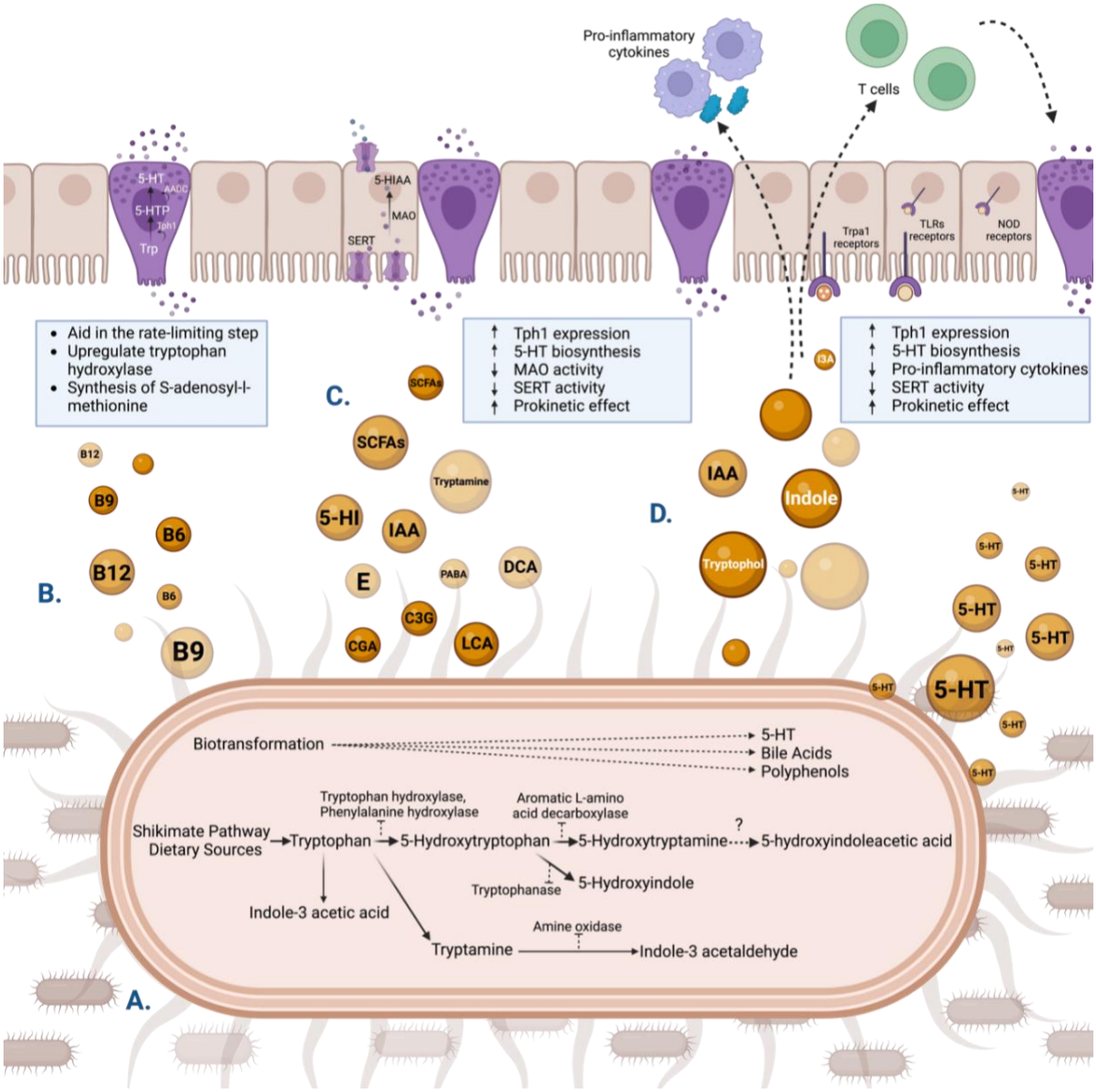

Figure 1.2. The gut microbiota modulate host serotonin (5-HT) levels through (A) direct synthesis of serotonin, metabolite synthesis, and deconjugation of 5-HT, bile acids, and polyphenols. Regulation of enterochromaffin (EC) cells through multiple pathways in the GI tract via (B) secretion of B vitamins; (C) secretion of SCFAs, tryptophan metabolites, secondary bile acids, and phenolic active compounds; (D) immune modulation. Abbreviations: SCFAs, Short-chain fatty acids; IAA, Indole-3-acetic acid; I3A, indole-3-carboxaldehyde; CGA, Chlorogenic acid; C3G, Cyanidin-3-glucoside; AADC, Aromatic L-amino acid decarboxylase; Trp, Tryptophan; 5-HI, 5-Hydroxyindole; TLRs, toll-like receptors; NOD, nucleotide oligomerization domain-like receptors; Trpa1, Transient receptor potential ankyrin A1; Tph1, Tryptophan hydeoxylase1; 5-HT, 5-Hydroxytryptamin; 5-HTP, 5-Hydroxytryptophan; SERT, Serotonin transporter; MAO, Monoamine oxidase; 5-HIAA, 5-Hydroxyindoleacetic acid; LCA, Lithocholic acid; DCA, Deoxycholic acid; PABA, p-aminobenzoate; E, Alpha-tocopherol.

5. Serotonin biosynthesis across the lifespan

Throughout the lifespan, similar to other organs, the gut microbiota undergoes drastic transformations, affecting many physiological processes, including serotonin biosynthesis. It is undeniable that prenatal stages form critical milestones, where fetal programming determines subsequent developmental and health outcomes [205]. Maternal health can substantially influence its trajectory as pregnancy is often marked by changes in metabolic, immunological, and hormonal functions. Microbial profiling during pregnancy shows a significant shift in bacterial load and composition, influenced by environmental factors, lifestyle, antibiotics, and dietary intake. Emerging evidence suggests a profound association between maternal microbial composition and fetal microbial colonization [206,207]. A recent study showed that probiotic supplementation during the last trimester induced significant changes in the taxonomic and functional composition of colostrum, biodiversity, and microbial interactions, resulting in a more complex microbial community [208]. Additionally, distinctly labeled bacteria introduced into the maternal gut have been detected in the intrauterine environment [209]. Despite earlier research by Collado et al. (2016) identifying consistent microbial communities in the placenta and amniotic fluid. While a comprehensive review by Kennedy et al. (2023) refuted the presence of stable microbial populations in the intrauterine environment [210,211]. Recent studies have also suggested that microbiota-derived extracellular vesicles may deliver microbial DNA and bacterial cargo, serving as a communication mechanism between the maternal microbiota and the fetus, as microbial extracellular vesicles have been detected in the intrauterine environment [212]. It is yet to be determined whether intrauterine microbes are involved in 5-HT synthesis or whether maternal-derived microbial extracellular vesicles play a role in the delivery and synthesis of 5-HT. Meanwhile, maternal microbiota seems to influence placental tryptophan and 5-HT availability [213], and disturbances in maternal microbial composition confer metabolic reprogramming

favoring the kynurenine metabolic pathway, reducing tryptophan availability for 5-HT metabolism [214]. High intake of dietary fiber during pregnancy has been reported to enhance colonic microbial composition, thereby promoting 5-HT metabolism, elevating maternal colonic and serum 5-HT levels, and increasing placental SERT expression and 5-HT uptake [215]. Maternal SERT is also essential in transporting 5-HT, as alterations in maternal SERT have been linked to a reduction in placental 5-HT levels and impairment in embryonic neuronal development, which is closely associated with autism spectrum disorder, whereas SSRI intake increases neurotrophic growth factor [216–219]. Furthermore, both the placenta and amniotic fluid were found to contain 5-HT derived from maternal sources, while the placenta can also synthesize 5-HT locally with the TPH1 enzyme, supported by maternal tryptophan uptake [220–222]. Throughout the gestational period, maternal tryptophan and 5-HT plasma levels show greater stability. With advancing gestation, placental tryptophan uptake increases together with 5-HT levels related to fetal 5-HT synthesis, exhibiting higher TPH activation in the fetal brain and intestine [223]. The importance of the gestational period in shaping the gut microbiota is consolidated in preterm infants, as they display a unique microbial composition compared to healthy term infants [224]. L-tryptophan and 5-HT remain significantly elevated up to 3 months of age in the plasma of preterm and term newborn infants with intrauterine malnutrition [225]. Given the potential neurotrophic role of serotonin in the fetal brain, any alteration in its metabolism during this critical period could lead to lasting changes in neuronal and immune development [225,226]. Beyond the gestational period, several developmental trajectories shape the microbial landscape, including the mode of delivery, early nutritional provision, geographical/culture factors, antibiotic use, and host genotype. Consequently, each infant undergoes unique microbial development during these early stages, as discussed extensively by Rinninella et al. (2019) and Martino et al. (2022) [227,228]. From the

time of birth, microbial composition experiences a gradual shift, generally characterized by an increase in alpha diversity and a decrease in beta diversity with an abundance of *Firmicutes* and a reduction of *Actinobacteria* and *Proteobacteria* [229]. Sanidad et al. (2024) found that human neonatal intestines harbor intestinal bacteria predominantly belonging to *Klebsiella grimintii, Clostridium perfringens, Staphylococcus aureus, Staphylococcus epidermis, and Enterobacter cloacae*, directly secrete serotonin and induce serotonin release from intestinal cells by upregulating *Tph1* and downregulating *Moa-a* [147]. Whereas 5-HT producing bacterial isolates from neonatal mice belong to *Rodentibacter heylii*, *Enterococcus gallinarum*, *Staphylococcus xylosus*, *Streptococcus acidominimus*, *Enterococcus faecalis*, and *Lactobacillus johnsonii* [147]. Likewise, gut microbiota isolated from piglets upregulated colonic *Tph1* expression and induced 5-HT secretion [230], suggesting that neonatal microbiota have the capacity to secrete and induce 5-HT synthesis. Although research in this domain is still in its infancy, both the gut microbiota and the 5-HT system undergo drastic changes throughout the lifespan, with a significant overlap in their developmental periods where mutual influence may occur [231]. During neonatal period, early colonization of the GI tract by intestinal microbiota influences the maturation and function of enteroendocrine cells responsible for producing most of the body's 5-HT, in turn 5-HT shapes gut development and motility, influencing microbial colonization [232]. Notably, high 5-HT concentration detected during the first two years of life, displaying a slow decline when it reaches an adult-like state by the age of five [233]. Similarly, the increase in intestinal microbial diversity stabilizes into an adult-like configuration around 3-6 years of age [227,228]. In nursing infants, breast milk is considered the primary source of tryptophan, which exerts antioxidant effects, and is a major source of 5-HT [234–236]. Formula feeding has been reported to reduce sow microbiota richness and diversity and shifted tryptophan colonic metabolism from 5-HT to tryptamine [237].

Additionally, postnatal maternal diet has been shown to affect 5-HT levels and microbiota composition in infants [238]. Transitioning into secondary succession in adolescence and adulthood, microbial composition become relatively stable dominated by *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*, while remaining malleable expressing circadian rhythms and diurnal cycles along with being heavily influenced by genetics, nutritional factors, antibiotic use, environment/geography, disease acquisition, sex hormones, and hormonal changes [227,228,239–241]. Research in adult populations suggests that, despite significant inter-individual variability in the gut microbiota, there may be a core microbiome at the functional level [242]. Studies indicate a similar trajectory for 5-HT, where 5-HT synthesis starts to stabilize at around 14 years of age and remains stable throughout adulthood [243]. Subsequently, serotonin synthesis and platelet/circulating 5-HT progressively decline with age, exhibiting statistically significant differences between extreme age groups with no sex differences [243,244]. During late succession, as the microbiome ages, alpha diversity decreases while beta diversity increases, leading to a distinct core microbial composition compared to secondary succession. This is characterized by a decline in microbial composition and commensal bacteria, with greater inter-individual variability influenced by various host-related factors. However, centenarians tend to retain a more diverse and beneficial microbiota that somewhat resembles that of their younger counterparts with distinct metabolic pattern [228,245,246]. Centenarians also harbor significantly more butyrate-producing bacteria associated with SCFAs metabolism and 5-HT synthesis [247]. Changes in microbiome diversity and composition in the elderly have been found to impair microbial metabolite synthesis including SCFAs and lead to serotonin-related diseases [86,248]. Ghare et al. (2022) observed an age-dependent increase in kynurenine and a substantial reduction in 5-HT synthesis owing to dysfunctional tryptophan metabolism and a decrease in butyrate-producing bacteria [249].

Interestingly, EC cells increase in aging populations, possibly as a compensatory mechanism to counteract the decline in serotonin levels [250].

6. Health disorders and serotonin deficiency

It has been stated that "serotonin is implicated in virtually everything, but responsible for nothing," highlighting the complex nature of serotonin's function across various systems [251]. In line with age-associated changes, numerous factors shape the gut microbiome throughout the lifespan. Although the definition of a healthy microbiome can vary, it is generally characterized by stability, diversity, resistance to stress, and a robust array of metabolic pathways [35]. It has been well-established that gut microbiome dysbiosis are associated with impaired tryptophan metabolism and serotonin biosynthesis, influencing myriad disease development and progression including neurological, psychiatric, immunological, and gastrointestinal disorders [167]. A fundamental element to this complex interaction is intestinal barrier, a selectively permeable structure composed of multiple components that function synergistically with the gut microbiota to regulate intestinal homeostasis. Nevertheless, various factors including genetics, diet, and environmental influences, can contribute to intestinal barrier impairment and gut dysbiosis, disrupting this delicate milieu [252,253]. An increase in intestinal permeability promotes widespread inflammation and facilitate the translocation of pathogenic microbes that thrive under dysbiotic conditions, shifting kynurenine to serotonin ratio toward the kynurenine pathway [254,255]. Disruption in gut homeostasis, along with alterations in 5-HT levels and signaling, are evident in patients with conditions such as IBD, IBS, and celiac disease, where the integrity and motility of the intestinal lining are compromised. These patients often experience shifts in microbial composition and tryptophan metabolism, favoring the kynurenine pathway, with increased activity of the enzyme indoleamine 2,3-dioxygenase and elevated kynurenine levels [256–259]. Notably,

the use of SSRIs has been shown to improve disease activity and reduce relapse rates under these conditions [260,261]. Given the high comorbidity of depression and other psychiatric disorders with intestinal diseases further underscores the intricate connection between the gut and serotonergic system [262]. Indeed, gut dysbiosis is known to reduce serum 5-HT levels and 5-HT/tryptophan availability, while promoting the kynurenine pathway. This shift, characterized by a reduction in serotonin and increase in kynurenine, is associated with neurodegenerative and neuropsychiatric disorders [263,264]. A common feature of neurodegenerative and neuropsychiatric disorders is poor sleep quality and sleep disturbances [265,266]. Serotonin helps sustain sleep architecture, depletion of gut microbiota through antibiotic treatment induces notable changes in the metabolism of amino acids and vitamins, including decrease in intestinal vitamin B6 and 5-HT concentrations [267]. Interestingly, the enrichment of gut microbiota, particularly with *Bifidobacterium* and *Odoribacter,* improved both sleep quality and 5-HT levels [268]. In addition, neurodevelopmental disorders such as ASD and Attention Deficit/Hyperactivity Disorder (ADHD) are associated with serotonergic impairment and microbial dysbiosis [269,270]. A significant portion of immune cells reside within the GI tract, forming an intricate connection with microbial residents [271]. Hence, the microbial community is linked to the onset and progression of various infectious and inflammatory diseases [272]. Viral infection and type I interferon-driven inflammation were found to reduce 5-HT levels by restricting tryptophan absorption, affecting its storage through platelet hyperactivation and thrombocytopenia, and enhancing MAO activity [273]. Various immune-related conditions, such as multiple sclerosis, epilepsy, and amyotrophic lateral sclerosis, are characterized by the infiltration of proinflammatory cytokines and alteration of 5-HT levels during microbial changes, while administration of SSRIs can exert anti-inflammatory and antioxidative effects [274–279]. Additionally, proinflammatory cytokines

induce the production of reactive oxygen species in macrophages, which in turn diminishes the BH4 co-factor, alters the serotonin synthesis pathway, and 5-HT levels by increasing the activity of SERT [113,280]. Elevated and dysregulated circulating 5-HT levels as observed with the use of SSRIs are associated with breast cancer, serotonin syndrome, osteoporosis, migraines, antidiuretic hormone irregularities, circulatory system disorders, and cutaneous conditions [281].

7. Modulation using nutritional factors

7.1. Fibers and prebiotics

Dietary fibers are classified into soluble and insoluble fibers depending on their water solubility, with insoluble fibers comprising the majority of total dietary fibers [282]. Insoluble fibers are mostly composed of carbohydrate polymers that can withstand digestive enzyme hydrolysis in the mammalian small intestine. In the large intestine, the microbiota initiates the fermentation process known as microbial hydrolysis, generating numerous bioactive metabolites that are critical for both humans and rodents [283]. Non-digestible carbohydrates are considered as major prebiotic and energy sources for gut microbiota. The fermentation of these prebiotic substances generate SCFAs [284]. Recently, microbial fermentation by-product SCFAs have been in the spotlight as their effects extend far beyond the GI tract by entering the circulatory system [285]. It is estimated that approximately 500-600 mmol of SCFAs are produced daily, which highly varies depending on the content of dietary fibers ingested, gut transit time, and status of gut microbiota [286]. SCFAs were found to modulate serotonergic function by regulating the expression and activity of SERT and 5-HTRs [287]. Additionally, increasing the availability of indigestible carbohydrates protects against tryptophan degradation, raises intestinal tryptophan levels, and promotes serotonin synthesis [11]. Remarkably, SCFAs can cross the blood-brain barrier, providing another possible avenue for modulating the serotonergic system [286]. Hart et al. (2024) observed high colocalization of SCFA

receptors in serotonergic neurons and microglia, depending on their amount and fiber solubility [288]. Evidence suggests that prebiotic fibers, such as oligosaccharides, ameliorate depression and anxiety-like symptoms via the serotonergic pathway. Oral supplementation with *Morinda officinalis* oligosaccharides increased TPH enzyme levels in the gut microbiota, enhancing bacterial 5-HTP synthesis. Despite 5-HTP synthesis, 5-HT levels in the gut are reduced [9]. Dietary fibers can direct tryptophan availability to more favorable tryptophan metabolites production by spore-forming bacteria associated with 5-HT synthesis [156,289]. Additionally, neoagaro-oligosaccharides mitigate gut dysbiosis and increase host serotonin levels. Fructo-oligosaccharide and probiotics regulate the hyper-serotonergic state in individuals with ASD, characterized by abnormal serotonin levels [290,291]. Most prebiotics derived from carbohydrates and non-carbohydrate oligosaccharides, including flavonoids and polyphenols stimulate lactic acid bacteria [292,293], which has been shown associated with the modulation of serotonin synthesis [294]. In addition, fermented dark tea with high polyphenol content promotes the abundance of serotonin-related gut microbiota, including *Akkermansia*, *Bacteroides*, and *Parabacteroides* [295]. In an AD mouse model, treatment with triphala polyphenols increased the abundance of bacterial phyla, particularly *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*, contributing to the enhancement of serotonin production, generation of SCFAs, reduction of neuroinflammation, and promotion of neurogenesis [296]. Wang et al. (2008) observed that the administration of corylin flavonoids transformed microbial diversity and composition, elevated 5-HTP levels in the colon, and reduced 5-HT; conversely, the expression of both 5-HTP and 5-HT in the CNS was elevated [297]. Similarly, quercetin and resveratrol improved alterations in the serotonergic system [298–301]. Studies have found that dietary phenolic compounds attenuate microbial dysbiosis, enrich beneficial microbiota, inhibit MAO, and reduce 5-HT degradation, which are subsequently

associated with the regulation of amyotrophic lateral sclerosis [302,302–305]. Conversely, dietary inulin is inversely correlated with 5-HT levels and increased abundance of lactic acid bacteria, while dietary restriction lacking prebiotics such as high-fat diet depletes microbial indole-3-acetate and tryptamine metabolites that act as stimulants for serotonin secretion from EC cells [306,307]. It has been postulated that diets rich in omega-6 fatty acids disrupt the intestinal microbial composition, which is ameliorated by omega-3 fatty acid supplements [308]. In fact, dietary consumption of omega-3 fatty acids favors the enrichment of butyrate-producing bacteria, which have been linked to the upregulation of *tph1* expression and intestinal 5-HT synthesis, acting as a protective buffer against stress and depression. Along with the enrichment of *Bifidobacteria*, *Lactobacilli,* and *Akkermanisa,* which exhibit similar serotonergic effects [309–315]. Reducing dietary omega-6 fatty acids has been correlated with a reduced risk of major depression, while omega-3 fatty acid deficiency increases serotonin turnover and decreases serotonin synthesis, release, receptor function and storage [316,317].

7.2. Probiotics and Postbiotics

Conventional treatments for serotonergic dysfunction have primarily relied on SSRIs; however, numerous studies have highlighted the drawbacks of their extensive use, including their effects on gut microbial communities and subsequent consequences on host health [318–320]. To a lesser extent, monoamine oxidase inhibitors (MAOIs) are also used as serotonergic treatments, even though their adoption is limited owing to potential side effects and safety concerns, despite current MAOIs being considered much safer than their first-generation counterparts [321]. Various serotonergic medications responsible for elevating serotonin levels have been associated with serotonin syndrome and unwarranted symptoms [322]. Most 5-HT secreted by EC cells is released basolaterally into neighboring intestinal epithelial cells, whereas some 5-HT is apically released

into the intestinal lumen, influencing microbial communication and homeostasis [323–327]. For instance, exposure of the probiotic *Enterococcus faecium* NCIMB10415 to 5-HT enhanced epithelial barrier function, increased biofilm formation, and adhesion to intestinal cells [328]. Certain spore-forming bacteria are known to induce 5-HT secretion from EC cells and are enriched by luminal 5-HT [329]. Fung et al. (2019) demonstrated that these spore-forming bacteria express 5-HT transporters homologous to the mammalian SERT, enabling the uptake of 5-HT by intestinal bacteria. This uptake can be altered by fluoxetine, leading to changes in bacterial gene expression and reduces colonization of spore-forming bacteria, thereby affecting the host intestinal gene expression related to lipid and steroid metabolism [329]. Additionally, several classes of SSRIs were found to exert antimicrobial activity against bacterial species [330]. Indeed, various antidepressant treatments (SSRIs, SNRIs, and MAOIs) were able to inhibit the growth of intestinal bacteria, some of which play essential role in peripheral 5-HT synthesis [294,331–333]. Correspondingly, treatments utilized to downregulate 5-HT levels such as antipsychotic and anticholinergic medications in individuals with abnormally high 5-HT concentrations have been shown to negatively impact intestinal microbial communities [21,322,334–337]. Therefore, there is a clear need to explore alternative treatments that can mitigate the side effects associated with current serotonergic medications. Growing scientific evidence on the multifactorial roles of probiotics, described as “live microorganisms when administered in adequate quantities, confer health benefits on host organisms”, has heightened consumer interest in probiotic products and supplements, driving innovative product development [338]. Along with the increasing prevalence of disorders related to serotonin dysregulation, researchers have explored more therapeutic applications of probiotics, particularly psychobiotics, which confer anxiolytic and antidepressant effects [339,340]. Besides, bacterial derived bioactive products or metabolic byproducts from

probiotic microorganisms known as postbiotics such as SCFA, vitamins, enzymes, proteins, and among other compounds, have shown the potential to retain antimicrobial, anti-inflammatory, antioxidative, immunomodulatory, and other beneficial physiological effects. Their ability to influence host systems without relying on the survival of live bacteria makes them promising candidates for therapeutic development [341]. Recent studies on the effects of gut-derived probiotics and postbiotics on serotonergic regulation and host health are summarized in Table 1.1.

Table 1.1. The Effect of probiotics/postbiotics on serotonergic system and their health outcomes

Intervention	Study Design	Mode of Regulation	Associated Health Outcome	Refs
Bifidobacterium infantis Bi-26*, Lacticaseibacillus rhamnosus* HN001*, Bifidobacterium lactis* BL-04*, and Lacticaseibacillus paracasei* LPC-37	Participants with ASD	Increased SCFAs and regulated 5-HT levels	Attenuated symptoms of ASD	[291]
Lactiplantibacillus plantarum 8PA3 *& Lacticaseibacillus rhamnosus* 12L	*In vivo*	Produced 5-HT & 5-HIAA, exerted antioxidant effects	Improved cognitive and behavioural impairments	[148]
Lactobacillus plantarum	*In vivo*	Increased 5-HT gene expression and 5-HT levels	Attenuated symptoms of Multiple-Sclerosis	[342]
Limosilactobacillus mucosae	*In vivo* (colitis mouse model)	Increased levels of indole-3-acetic acid, while decreasing colonic 5-HT	Alleviated colitis associated symptoms	[343]
Akkermansia muciniphila	*In vitro & In vivo*	Induced serotonin synthesis by upregulating TPH1 and SERT expression	Modulated GI motility and restore gut microbiota	[344]
Bifidobacterium longum CCFM1029	*In vivo & Clinical Trails*	Upregulated Tryptophan metabolism and modulated immune responses	Alleviated atopic dermatitis related pathologies	[345]
Bifidobacterium animalis subsp *lactis* LKM512	Patients with atopic dermatitis	Elevated the expression of kynurenic acid metabolites	Alleviated atopic dermatitis related pathologies	[346]
Escherichia coli Nissle 1917	*Ex vivo* (mouse ileal tissue)	Elevated 5-HT bioavailability and intracellular 5-HTP, while reduced 5-HIAA levels	ND	[347]

Bifidobacterium dentium	*Ex vivo*	Increased acetate and 5-HT concentrations, upregulated the expression of SERT in ileum and colonic tissue and hippocampal 5-HTRs	Decreased repetitive and anxiety-like behaviours	[348]
Lactobacillus rhamnosus GG	*In vitro* & *In vivo*	Restored low SERT expression & 5-HT transporter protein	Modulated IBS symptoms	[349]
Multi-Probiotics supplement (*Bifidobacterium longum*CCFM687, *Bifidobacterium breve* CCFM1025, and *Pediococcus acidilactici* CCFM6432)	MDD Patients	Increased SCFAs and colonic 5-HT levels, ameliorated *Slc6a4* over-expression	Alleviated depressive symptoms and gastrointestinal dysfunction	[350]
L. acidophilus (DSM 20079)	*In vitro* (acetic acid-induced colitis model)	Reduced abnormal serotonin levels associated with colitis	Ameliorated IBD related pathologies	[351]
Bifidobacterium breve M2CF22M7 and *Bifidobacterium longum subsp. infantis* E41	*In vitro* & *In vivo* (depression animal model)	Increased Tph1 expression and 5-HTP secretion from RIN14B cells with no impact on 5-HT levels, along with SCFA enrichment and reversed 5-HT and 5-HTP deficiency in mice	Substantial reduction in depressive behaviours	[352]
Bifidobacterium breve CCFM1025	*In vitro* & *In vivo* (Chronically stressed model)	Ameliorated gut dysbiosis, increased intestinal 5-HTP and SCFAs, while hippocampal 5-HT levels were elevated without changes in TPH2 & *slc6a4* activity	Reduced HPA axis hyperactivity, depression- and anxiety-like behaviours	[353]
Bifidobacterium animalis F1-7	*In vivo*	Promoted 5-HT synthesis, upregulated 5-HTRs and TPH expression, while downregulating SERT	Alleviated intestinal peristalsis	[354]
Limosilactobacillus fermentum SLAM 216 (LF216EV)	*In vitro & In vivo*	Elevated expression of 5-HT related genes (htrb2c, sert, and tph-1) in the skin and gut, as well as elevated 5-HT serum levels	Alleviated atopic dermatitis related pathologies	[355]
Escherichia coli Nissle 1917	*In vitro* (IL-1β-induced inflammation model)	Decreased free 5-HT levels by upregulating SERT expression	Therapeutic intervention for IBD related pathologies	[356]

ND: Not Determined

8. Future perspectives

As our understanding of the intricate connection of the gut-brain axis continue to evolve, the need for public health policies and public awareness campaigns aimed at promoting gut health and gut-supportive lifestyle practices through healthcare implementation, schools, community-based workshops become increasingly important. To sustain balanced 5-HT levels, it is crucial to maintain a resilient gut microbiota that can effectively respond to stress, diet, and other environmental stimuli. Prebiotics and probiotics as discussed, when integrated into our daily diet, have the potential to restore gut microbial balance, promote serotonin synthesis in the gut, and improve both physical and mental health outcomes. Research continues to advance in different delivery methods and applications of specific probiotic strains and prebiotic supplements that may be most effective in restoring and maintaining serotonergic homeostasis. Postbiotics, in particular, offer advantages such as improved stability and longer shelf life, making it suitable for pharmaceutical applications [357]. A personalized approach to treating serotonin-related conditions by assessing gut health and microbial biomarkers might be beneficial, as recent advances in microbiome-based research suggest that targeted modulation of the gut microbiota could significantly impact individual health outcomes [35]. Microbiome-based therapies, such as prebiotics, probiotics, and postbiotics, hold promise for precisely managing gut-related disorders. In-depth profiling of an individual's gut microbiota using advanced technologies can guide the development of tailored microbiome-based therapies, potentially enhancing serotonin regulation and serotonin-related conditions. However, personalized microbiome interventions face challenges, including individual variability in response, safety concerns, regulatory hurdles, accessibility, and data privacy [357]. Despite growing evidence supporting the benefits of prebiotics and probiotics, they face challenges against regulatory frameworks. European regulatory

authorities require robust clinical evidence and validated biomarkers for any health claims. While some prebiotic supplements, such as chicory inulin, have been approved for bowel function claims, broader acceptance remains limited. Other leading countries including Canada, USA, Japan, and China tend to have more flexible regulations, allowing functional health claims on prebiotic-enriched products. Similarly, global regulations of probiotics are fragmented and lacks consistency [358,359]. Addressing these issues through ongoing research and collaboration will be essential to fully realize the potential of microbiome-based personalized treatments for serotonin-related and other health conditions.

9. Conclusion

The gut microbiome plays a pivotal role in serotonin biosynthesis through both direct and indirect pathways, influencing peripheral serotonin levels throughout life. This relationship highlights the importance of maintaining a healthy gut microbiome for optimal serotonin function and overall health. Emerging evidence supports the notion that disturbances in the gut microbial composition can lead to serotonergic impairment, contributing to various health disorders. Given the significant impact of gut microbiota on serotonin regulation, further research is warranted to explore therapeutic strategies that target the gut microbiome. Specifically, investigating the effects of probiotics, prebiotics, and other dietary interventions on gut microbiota composition and their subsequent influence on serotonin levels could offer promising avenues for the treatment of serotonin-related disorders. Recognizing the role of gut microbiota in serotonin regulation supports the inclusion of prebiotic- and probiotic-rich foods in dietary recommendations to promote mental and gastrointestinal health. Additionally, longitudinal studies examining changes in the gut microbiome and serotonin biosynthesis across different stages of life could provide deeper insights into their dynamic relationships. Overall, advancing our understanding of the gut microbiome–

serotonin axis holds great potential for developing novel therapeutic approaches to improve mental and gastrointestinal health.

Author contributions

SA, SM, and RH were involved in the conceptualization and design of the article. SA drafted the initial version of the manuscript. SM and RH reviewed and revised the draft. All authors contributed to the article and approved the submitted version.

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10. Statement of the research problem, rationale, and objectives

We hypothesized that a healthy gut microbiome contains bacterial strains that can directly synthesize serotonin or induce its production from host enterochromaffin cells. This research project aims to explore the direct implication of gut microbiota in serotonin biosynthesis in the gastrointestinal tract and identify potential gut probiotic candidates. The specific objectives of the MSc project are:

1. Screen bacterial strains obtained from healthy gut microbiome for tryptophan hydroxylase-1 enzyme and quantify serotonin production in the identified bacterial strains.
2. Analyze the identified bacterial strains for the induction of serotonin synthesis in enterochromaffin cells.
3. Evaluate the potential probiotic properties of bacterial strains that produced or induced high quantities of serotonin.

CHAPTER 2:

Screening, characterization, and host modulation of 5-hydroxytryptophan-producing potential probiotic candidates from human gut microbiota

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Abstract

Ample research suggests a dynamic connection between the serotonergic system and the gastrointestinal (GI) tract, with the gut microbiota emerging as a key regulator of host serotonin biosynthesis in the GI tract. However, the precise influence of healthy gut microbiota on serotonin (5-hydroxytryptamine; 5-HT) synthesis has not yet been fully elucidated. Here, we investigated the contribution of gut microbiota to 5-HTP synthesis, an intermediate 5-HT precursor. Through isolation of bacterial strains from healthy donors, screening of TPH enzyme activity, and confirming 5-HTP secretion via LC–MS and ELISA. To further examine bacterial 5-HT induction, enterochromaffin RIN14B cells were exposed to selected bacterial strains with high enzymatic activity and probiotic potential. Our results identified considerable variability among the major gut microbial phyla, with *Bacillota* having notably higher TPH enzyme activity. *Bifidobacterium*, *Lactobacillus, Enterococcus, Staphylococcus*, *Streptococcus*, *Acidaminococcus*, and *Collinsella* have been identified as key contributors to serotonin precursor synthesis at the genus level. Selected strains *Lacticaseibacillus rhamnosus* UO.H3002 and *Bifidobacterium longum* subsp. *Suillum* UO.H1105 with high enzymatic activity and 5-HTP secretion demonstrated promising probiotic traits. These findings suggest that targeted modulation of 5-HT biosynthesis through specific gut microbes offers potential therapeutic avenues to increase host 5-HT levels via enhanced 5-HTP secretion, thereby providing new insights into the impact of the gut microbiome on host 5-HT regulation.

Keywords: Serotonin, 5-hydroxytryptophan, Gut microbiota, Tryptophan hydroxylase, Probiotics, Gut-Brain axis.

1. Introduction

The mammalian intestine harbors more than trillions of microorganisms, including bacteria, viruses, fungi, archaea, and protozoa, collectively known as the gut microbiota. These microorganisms play essential roles in host physiological processes and pathophysiological progression [360,361]. Emerging evidence suggests that the gut-brain axis, mediated by the gut microbiota, regulates host physiology and is implicated in neuropsychiatric conditions [256]. Alterations in gut microbiota composition and metabolism are linked to several serotonergic-related disorders [362,363]. One of the most recent core discoveries in microbiome research is its influence on the biosynthesis and function of neuroactive metabolites, including γ-aminobutyric acid (GABA), dopamine, norepinephrine, and serotonin [364–366].

Serotonin (5-hydroxytryptamine; 5-HT) is an evolutionarily conserved molecule often referred to as the "feel-good" neurotransmitter or "happiness hormone", found in various species ranging from nematodes to humans. Serotonin biosynthesis pathway involves the conversion of tryptophan to 5-hydroxytryptophan (5-HTP) by the rate-limiting enzyme tryptophan hydroxylase (TPH) [43]. 5-HTP is then converted to serotonin by aromatic L-amino acid decarboxylase (AADC) [43]. Approximately 90% of the body's total serotonin is produced in the gut, primarily by enterochromaffin (EC) cells, through dietary tryptophan metabolism [11]. Serotonin is recognized for its essential role in cognition and emotion regulation along with various physiological processes including intestinal motility, homeostasis, immune signalling, and metabolism [13,27,67,135,367]. Similarly, the precursor 5-HTP is involved in the regulation of many psychological and physiological functions, and its supplementation is often effective in treating several conditions, such as depression, migraines, pain, appetite, and insomnia [43,368].

Emerging evidence demonstrates the substantial influence of gut microbiota on serotonin biosynthesis and regulation [32,256]. For instance, germ-free mice have substantially reduced

serum serotonin levels and colonic *tph1* mRNA expression along with increased serotonin-selective reuptake transporter mRNA expression compared to control mice [369]. Certain lactic acid bacteria were found to increase *tph1* expression of in RIN-14B cells and stimulate the secretion of 5-HTP and 5-HT [294]. Likewise, *Akkermansia muciniphila* stimulated *tph1* gene expression in EC cells and decreased the expression of *SERT* in Caco-2 cells [344]. Similarly, *Bifidobacterium longum* N61 isolated from infants promoted serotonin secretion by RIN-14B cells [370].

While most research has focused on indirect modulation of host serotonin production, direct microbial synthesis pathways are largely unexplored. For instance, Sanidad et al. [147] reported direct synthesis of 5-HT from neonatal gut microbiota, whereas Zhang et al. [9] suggested a putative serotonin synthesis pathway in gut bacteria initiated by 5-HTP synthesis via the TPH enzyme. Additionally, studies have indicated the presence of several TPH rate-limiting co-factors in intestinal bacteria, including BH_4 and S-adenosyl-l-methionine [120,151]. Although the precise biosynthetic mechanisms in gut microbiota remain unclear, two potential metabolic pathways have been proposed: plant-like pathway that involves the decarboxylation of tryptophan to tryptamine followed by hydroxylation and an animal-like pathway, which entails hydroxylation of tryptophan to 5-HTP followed by decarboxylation [10]. A previous study reported that nearly 20% of gut-associated integrated microbial metagenomes are represented by an animal-like pathway, whereas the plant-like pathway are rare [157]. Despite these advances, the relative contributions of gut microbiota to the overall production of serotonin in the intestine are yet to be fully elucidated. This study aimed to screen various intestinal microbial taxa representative of healthy gut microbiota for TPH activity, assess their capacity to induce serotonin synthesis in ECs, and their probiotic properties.

2. Materials and Methods

2. 1 Isolation of bacterial strains

Strains were isolated from a fecal sample collected from a healthy female and male donors in Ottawa, Canada, with the approval from the University of Ottawa Research Ethics Board (certificate H-02-18-347). Fecal samples were homogenized with reduced peptone water (20%, w/v). Homogenized samples were then serially diluted (10^{-1} to 10^{-8}) with peptone water and spread onto various (1%) agar-supplemented culture media: brain heart infusion broth supplemented with yeast extract, cysteine, vitamin K1, hemin, and 1% trace vitamin solution; fastidious anaerobic broth supplemented with 0.5% yeast extract and 10 mM glutamic acid; Gifu anaerobic broth; and yeast casitone fatty acids broth. Plates were incubated under anaerobic conditions (85% N_2, 10% H_2, and 5% CO_2) for five days. Subsequently, colonies were isolated and streaked on their respective agar plates cultivated for 72 h at 37°C in an anaerobic chamber, from and single colonies from each plate were then inoculated into their respective culture broth for 72 h at 37°C in an anaerobic chamber and preserved in 50% glycerol at -80 °C. All strains used in this study and their growth conditions are listed in **table 2.S1**.

2.2 DNA extraction, 16S rDNA-based characterization, and taxonomic assignment

DNA was extracted from bacterial isolates after cultivation in the late exponential phase, and the pellet was collected by centrifugation using the NucleoSpin Microbial DNA kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's protocol. Concentration and quality were assessed using a Nanodrop (Thermo Scientific NanoDrop 2000, USA). For 16S rRNA gene amplification, PCR was performed using universal primers 8F and 1391R [371]. PCR reaction mixture included 1× PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs (Invitrogen, Carlsbad, CA, USA), 1 μM of each primer, 1U of Taq DNA polymerase (Invitrogen), and 50-100 ng of bacterial

DNA in a total volume of 50 µL of nuclease-free water. The PCR conditions were as follows: initial denaturation at 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, with a final extension at 72 °C for 5 min. PCR amplifications were verified by gel electrophoresis on 1.2% agarose gel stained with ethidium bromide [372]. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced at the Ottawa Hospital Research Institute's DNA sequencing facility (Ottawa, ON, Canada). The quality of the chromatograms was assessed using BioEdit v7.7. Taxonomic identification was performed using the RDP classifier 27 with a nucleotide similarity threshold of 99% for the 16S rRNA gene to determine species-level identification, and was confirmed through BLAST analysis on the NCBI platform [373].

2.3. Preparation of bacterial lysates and supernatant

A total of 82 bacterial strains representative of the normal gut microbiota used for the experiment, consisting of previous and newly (this study) lab-isolated collection strains (N*u*GUT Research Platform, uOttawa) and strains purchased from the American Type Culture Collection (Table 2.S1), were cultured until late exponential phase in their respective media and growth conditions (Table 2.S1) supplemented with 1mM of L-Tryptophan (Thermo Fisher, USA). Supernatants were then collected after centrifugation at 8000 × g for 10 min, filtered using a 0.22 µm sterile syringe filter, and stored at -20°C. For lysate preparation, pellets were washed once with PBS and incubated for 15 min without heating in an ultrasonic bath (FALC INSTRUMENTS, Italy). Following cell lysis, the bacterial solution was centrifuged at 8000 × g for 10 min, and the resulting supernatant was filtered using a 0.22 µm sterile syringe filter and stored at -20 C.

2.4 Enzymatic assay for TPH1 Activity

Conditions for enzymatic activity were based on a previously established methodology by [374]. Briefly, the reaction was performed in a standard mixture containing 100 mM MES (pH 7.0), 400 mM ammonium sulfate, 2.0 mM dithiothreitol (DTT), 50 µg/mL catalase, 50 µM ferrous ammonium sulfate, 120 µM tryptophan, 600 µM (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4), 10 mM hydrochloric acid, 12.0 mM dithiothreitol and ddH_2O. The reaction was initiated by adding bacterial lysate to the standard mixture, and tryptophan hydroxylase activity was assessed by measuring 5-HTP concentration produced through the hydroxylation of tryptophan at excitation and emission wavelengths of 300 nm and 330 nm for 10-minute intervals over a 2-hour period. Assays were conducted in duplicate. Tryptophan hydroxylase activity was calculated based on the total protein concentration which was determined using the Bradford assay. The test was performed in duplicate.

2.5. Cell Culture and Bacterial Stimulation

The rat pancreatic cell line RIN14B (ATCC Cat# CRL-2059, RRID: CVCL_3583) was cultured in humidified incubator at 37 °C with 5% CO2 in RPMI 1640 medium, supplemented with 10% fetal bovine serum. The cells were seeded into 24-well plates at a density of 2×10^5 cells per well, grown for 72 h to sub-confluence in RPMI 1640 medium without phenol red and fetal bovine serum [333,375]. After 72 hours, cells were washed with phosphate-buffered saline containing 0.2% BSA and 2 µM fluoxetine (Enzo life Sciences Inc., USA), and incubated for 1 h with bacterial supernatant [333]. Negative controls were treated with cell medium and bacterial medium. After 1 hour incubation, the supernatants were collected, centrifuged at 6000 × g for 5 min, and frozen for subsequent 5-HT assays. The remaining adherent RIN14B cells were lysed using TRIzol reagent (Invitrogen, CA, USA). The test was performed in triplicate.

2.6. RNA Extraction and Quantitative real-time PCR (qRT-PCR)

Extracted cellular RNA was purified using the Invitrogen™ TURBO DNA-free™ Kit (Thermo Scientific, USA) and cDNA was amplified using the iScript Reverse Transcription Supermix kit (Bio-Rad, USA). The expression levels of *Tph1* mRNA were analyzed using quantitative real-time PCR (qRT-PCR) with SYBR Green (Bio-Rad, USA). The primers pairs used were *Tph1* (F-5′-GGCTCTGGTTCTGCGATATT-3′, R-3′-TTGAGTCTGTCCTGGTGTTTG-5′) and glyceraldehyde 3-phosphate dehydrogenase (Gapdh) (F-5′-AGGTCGGTGTGAACGGATTTG-3′, R-3′-TGTAGACCATGTAGTTGAGGTCA-5′). PCR amplification involved an initial pre-denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Melting curve analysis was performed over a 65–95 °C range with a 0.5 °C ramp rate. All reactions were performed in triplicate. The relative expression of *Tph1* mRNA was normalized to Gapdh using the $2^{-\Delta\Delta CT}$ method [376].

2.7 Quantification of 5-HT and 5-HTP

2.7.1. Metabolomic quantification by nano-flow LC–MS/MS

For 100 µL of bacterial CFS, 900 µL of methanol (−20°C) and 1 µL of reserpine (internal standard, Sigma-Aldrich) were added and the mixture was then chilled at −20°C for 15 min, thawed, vortexed, and centrifuged at 12,000 × g for 5 min. The supernatant was dried (SpeedVac system), re-dissolved in 100 µL of 50% acetonitrile, 50% water, and 0.1% formic acid, and filtered (0.2 µm PTFE filter). Samples were then collected in LC vials and analyzed by nanoLC coupled to a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific). Proxeon EASY nLC II System with a Thermo Scientific Acclaim PepMap RSLC C18 column was used to separate metabolites. Neuroactive metabolites were identified by comparing m/z values and retention times with standards with 6-point calibration curve (1 to 1000 µM), and low-intensity peaks were excluded.

Data were processed with ZMine 2.53, and tandem MS scans were analyzed using SIRIUS by matching results to PubChem organic formula database. Identified metabolites were cross-referenced with HMDB and MiMeDB databases [377,378]. MetaboAnalyst was used for PCA and heatmaps analysis [379,380]. The tests were performed in triplicate.

2.7.1. ELISA assays

5-HTP quantification was confirmed using a competitive inhibition enzyme-linked immunosorbent assay 5-HTP (ELISA) kit (MyBioSource, California, USA). Briefly, to 50 µL of sample and standards, 50 µL of detection reagent A was added to each well and incubated for 1 hour at 37°C. Each well was aspirated and washed with wash solution three times. After removing any remaining wash solution, 50 µL of detection reagent B was added to each well and incubated for 30 min at 37°C, aspiration and washing process repeated five times. To each well 90 µL of substrate solution was added and incubated for 10-20 min at 37°C, to stop the reaction 50 µL of stop solution was added. Absorbance was measured at 450nm. The tests were performed in duplicate.

2.8 Evaluation of Probiotic Properties

2.8.1 Auto-aggregation Assay

Auto-aggregation of the selected potential probiotic candidates was assessed following the method outlined by Jena et al. [381]. Briefly, selected strains were grown overnight at 37°C in deMan, Rogosa, and Sharpe (MRS)+0.05%L-cystein media , centrifuged and washed twice with PBS (pH 7.0) before resuspension in PBS. The optical density (OD) of the bacterial suspension was standardized to 0.25 ± 0.05 at 600 nm, corresponding to a concentration of 10^8 cfu/mL. Bacterial suspensions were then incubated at 37°C for varying time intervals (0, 2, 4, 16, and 24 h).

Lacticaseibacillus rhamnosus GG was used a positive control [382]. The test was performed in duplicate. The auto-aggregation percentage was calculated from the absorbance at 600 nm using the following equation: Auto-aggregation (%) = $(A_0 - A_t)/ A_0 \times 100$

2.8.2 Antibiotic Susceptibility Assay

Antibiotic susceptibility tests were conducted by testing selected strains against various antibiotics, including gentamicin (VWR, New York, USA), ampicillin, vancomycin, gentamicin, streptomycin, erythromycin, tetracycline, and chloramphenicol (Alfa Aesar, Mississauga, Canada) [381,383]. One hundred microliters of each antibiotic solution (1 mg/mL) were added to 100 µL of MRS medium supplemented with L-cysteine, followed by serial dilutions in a 96-well flat-bottom microplate. Each well was inoculated with 100 µL of the selected potential probiotic candidates after optical density of the bacterial suspension at 600nm was adjusted to 0.25 to standardize bacterial concentrations (10^8 CFU/mL). The test was performed in duplicate, and the absorbance at 600 nm was measured during 24 h of incubation at 37°C using a Tecan microplate reader (Grödig, Austria). The minimum inhibitory concentration (MIC) was determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines for of *L. rhamnosus* UO.H3002 [383,384] and the EFSA guidelines for *B. longum subsp. suillum* UO.H1105 as CLSI guidelines are not currently available for *Bifidobacterium* [385].

2.8.3 Resistance to Stimulated Gastric and Intestinal Fluids

The method used to evaluate the survivability of probiotic candidates under gastrointestinal conditions was adapted with slight modifications from [386]. Cultures of the probiotic candidates were grown at 37°C for 24 h, centrifuged at 8000 × g for 5 min, and were washed twice with phosphate buffered saline (PBS, pH 7.2, 10 mM). The pellet was resuspended in 10 mL of MRS

broth. This suspension was combined with an equal volume of a simulated saliva-gastric solution, which contained $CaCl_2$ (0.22 g/L), NaCl (16.2 g/L), KCl (2.2 g/L), $NaHCO_3$ (1.2 g/L), and 0.3% (w/v) pepsin (Sigma, Canada). Samples were taken immediately after inoculation in saliva-gastric solution and serially diluted to measure the initial cell count (before adjusting the pH to 2.5 with 6M HCl) using colony counting technique. After adjusting the pH, samples were incubated for 120 minutes at 37°C which were subsequently serially diluted, and cell count were measured using plate drop method. The remaining initial bacterial cultures were centrifuged again at 8000 × g for 5 min and the pellet was resuspended to the original volume in their respective medium broth containing 0.3% (w/v) bile salts and 0.1% (w/v) pancreatin (Sigma, Canada) at pH 7.5; viability was assessed after a 180-min incubation at 37°C by serial dilution and plate drop method. The test was performed in duplicate.

2.9 Statistical Analysis

Data analysis performed in R (v. 4.3.1) using RStudio 2023.06.0 Build 421. Heatmap was created with the *ComplexHeatmap* (v.2.16.0) and circlize (v.0.4.16) packages [387,388]. Principal component analysis (PCA) was conducted and visualized with the *factoextra* (v.1.0.7) [389] and *ggplot2* (v.3.5.1) [390] packages. PERMANOVA with 999 permutations was performed using the Adonis2 function from the vegan package (v2.6.6.1) [391]. A one-way ANOVA was conducted to examine variation in TPH1 production between strains at the phylum and genus levels using the *welch_anova_test* function from the *rstatix* package (v.0.7.2). Pairwise comparisons between taxa were conducted using the *tukey_hsd* post hoc test to determine significant differences within groups, utilizing the same package [392]. Results were considered significant at an adjusted *p*-value of < 0.05.

3. Results

3.1 Quantification of tryptophan hydroxylase activity in gut microbes

A collection of 82 bacterial strains representative of normal gut microbiota was assessed for tryptophan hydroxylase (TPH) activity as shown in (**Figure 2.1**), which illustrates the diversity of TPH activity among these strains. Notably, species such as *Collinsella aerofaciens* UO.H1074 (6562.4 ± 2683 U/mg), *Enterococcus faecalis* UO.H3013 (1513 ± 288 U/mg), *Streptococcus samguinis* ATCC BAA-1455 (1380 ± 99 U/mg), *Bifidobacterium bifidum* UO.H1106 (1253 ± 76 U/mg), *Lacticaseibacillus rhamnosus* UO.H3002 (185 ± 74 U/mg), and *Bifidobacterium longum* subsp. *suillum* UO.H1105 (145 ± 18 U/mg) demonstrated the highest TPH activity levels. Conversely, *Phocaeicola massiliensis* UO.H1001 (0.8 ± 0.12 U/mg) showed minimal to no TPH activity. Clustering within the heatmap (**Figure 2.1**) highlights species with similar TPH activity, with lactic acid bacteria including *Bifidobacterium*, *Enterococcus*, and *Lactobacillus* forming distinct functional groups with varying high levels of activity, in addition to *Staphylococcus* strains such as *Staphylococcus simulans* UO.H3021 (680 ± 203 U/mg) and *Staphylococcus sp.* UO.H3024 (704 ± 132 U/mg). This clustering provides valuable insights into shared metabolic functions among different microbial groups, as indicated by their similar levels of TPH activity.

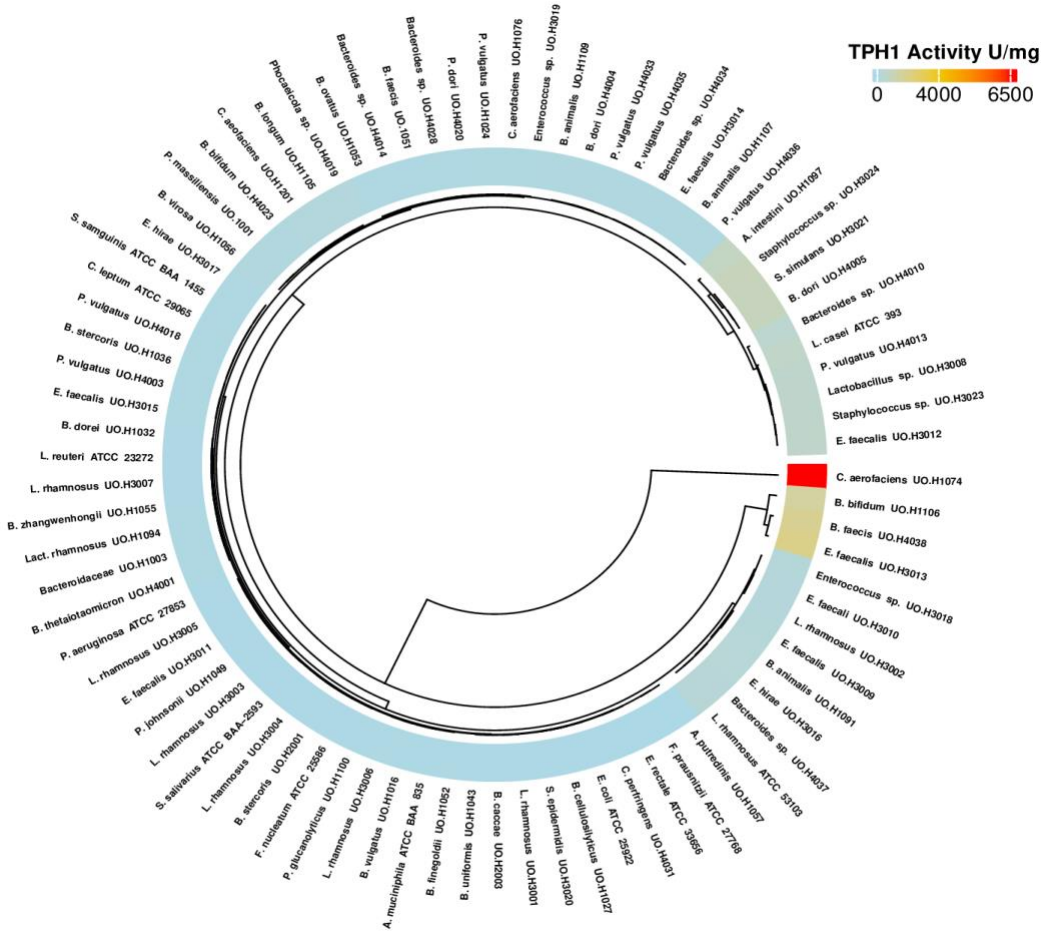

Figure 2.1. Heatmap of TPH activity (U/mg) across gut microbial species.

Further investigation into the functional diversity among genera through Principal Component Analysis (PCA) analyzing gut bacterial genera with respect to their TPH activity (**Figure 2.2A**). This figure displayed a statistically significant separation between bacterial genera, indicated by an R^2 value of 0.25 ($p = 0.007$). The distinct levels of PC1 and PC2 across the seven groups accounted for 39.6% of the total variation and demonstrated genus-specific clustering patterns. *Staphylococcus*, *Lactobacillus*, *Bacteroides*, *Bifidobacterium*, and *Enterococcus* formed closely related clusters, suggesting functional homogeneity within these groups. In contrast, *Collinsella* and *Phocaeicola* appeared more dispersed on the plot, indicating greater variability in their functional profiles.

A comparison across all genera representative of gut microbiota indicated wide variability in TPH activity among 20 bacterial genera **(Figure 2.2B**). *Collinsella* showed the highest TPH activity (2260 ± 3726 U/mg), followed by *Streptococcus* (888 ± 704 U/mg) and *Staphylococcus* (463 ± 319 U/mg) among various other bacterial genera, including *Bifidobacterium* (309 ± 416 U/mg), *Enterococcus* (284 ± 427 U/mg), and *Lactobacillus* (135 ± 175 U/mg), while indicating greater in-group variability suggesting strain-dependent activity.

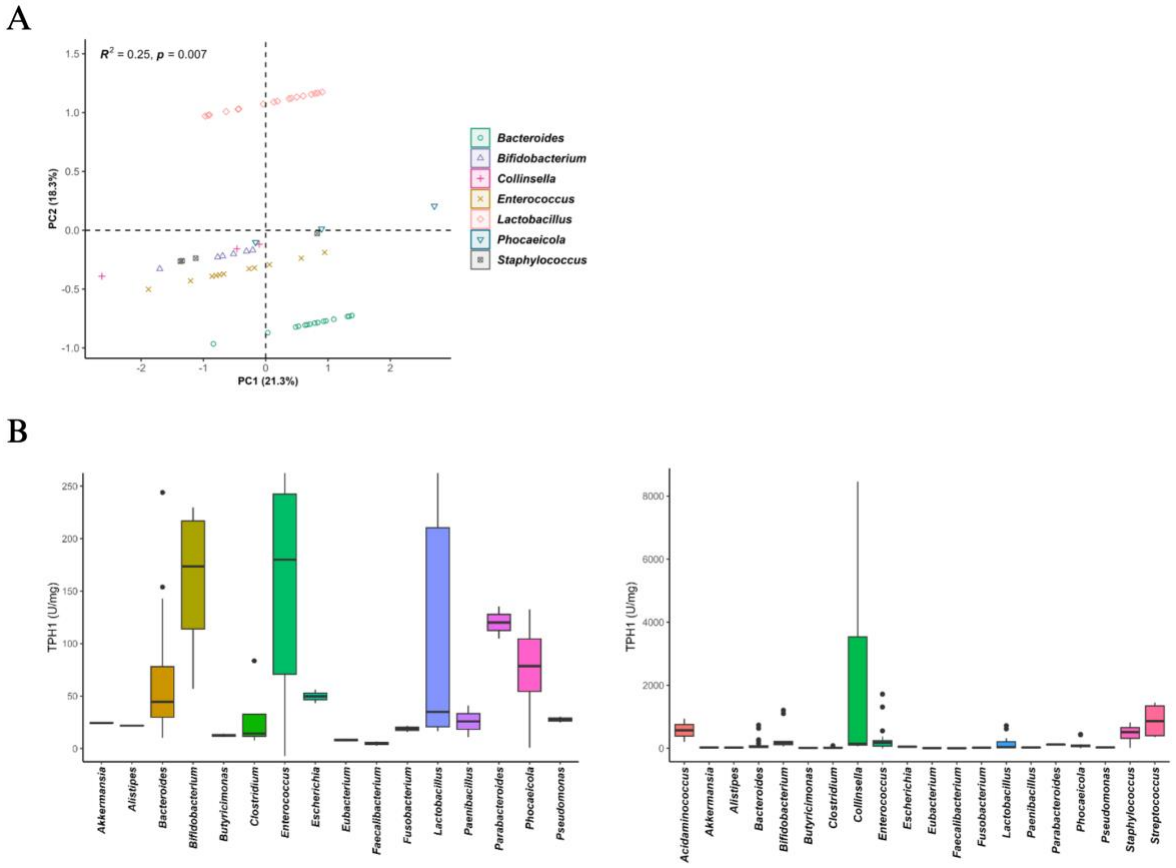

Figure 2.2. Analysis of gut microbial genera based on TPH activity. (A) principal component analysis (PCA) of gut bacterial genera (R^2 = 0.25, p = 0.007). (B) Comparison of TPH Activity (U/mg) across genera representative of gut microbiota.

Statistical analysis shown in **Figure 2.3B,** confirms a significant difference between dominant phyla, particularly between *Bacillota* and *Actinobacteria* ($p < 0.05$), and *Bacillota* and *Bacteroidota* ($p < 0.05$). Notably, *Bacillota* exhibited substantially higher TPH activity compared

to both *Actinobacteria* and *Bacteroidota*. At the genus level shown in **Figure 2.3A**, *Bifidobacterium* and *Lactobacillus*, exhibited among the highest levels of TPH activity. *Enterococcus* also demonstrated elevated activity; however, the presence of several notable outliers within this genus indicates considerable intra-genus variability.

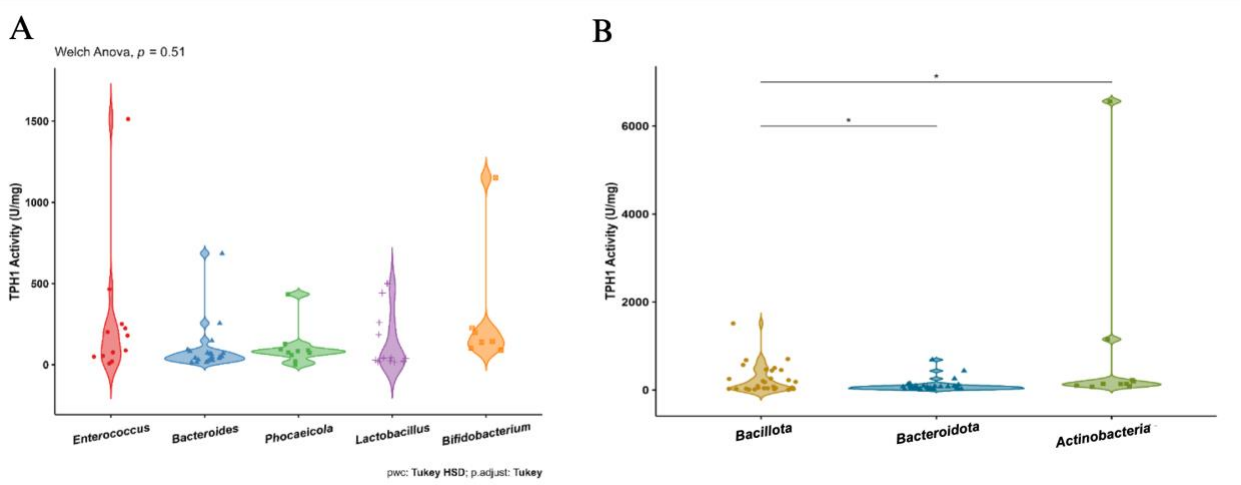

Figure 2.3. Statistical analysis of TPH activity among the dominant (A) genera and (B) phyla in healthy gut microbiota residents. *p*-value of < 0.05.

3.2 Production of neuroactive metabolites by selected potential probiotic candidates

Based on tryptophan hydroxylase activity, potential commensal strains with the highest enzyme activity were selected to screen for metabolite secretion as shown in (**Figure 2.4 A, B**). Metabolomics analysis did not detect significant levels of 5-HTP secretion in the bacterial supernatants of *Lacticaseibacillus rhamnosus* UO.H3002 and *Bifidobacterium longum subsp. suillum* UO.H1105. However, both strains substantially secreted GABA, with respective concentrations of 135.4 ± 14.2 µM and 638.4 ± 80.1 µM. Due to the detection limits of LC-MS/MS metabolomics (LOD = 1 µM), an ELISA assay was used to confirm 5-HTP secretion in selected bacterial strains. CFS from *B. longum subsp. suillum* UO.H1105 (22.2 ± 31 nM) exhibited the

highest 5-HTP concentration, followed by *Lactobacillus sp.* UO.H3008 (49.6 ± 18 nM), *L. rhamnosus* UO.H3002 (1.6 ± 1.2 nM), and *B. bifidum* UO.H1106 (1.6 ± 0 nM).

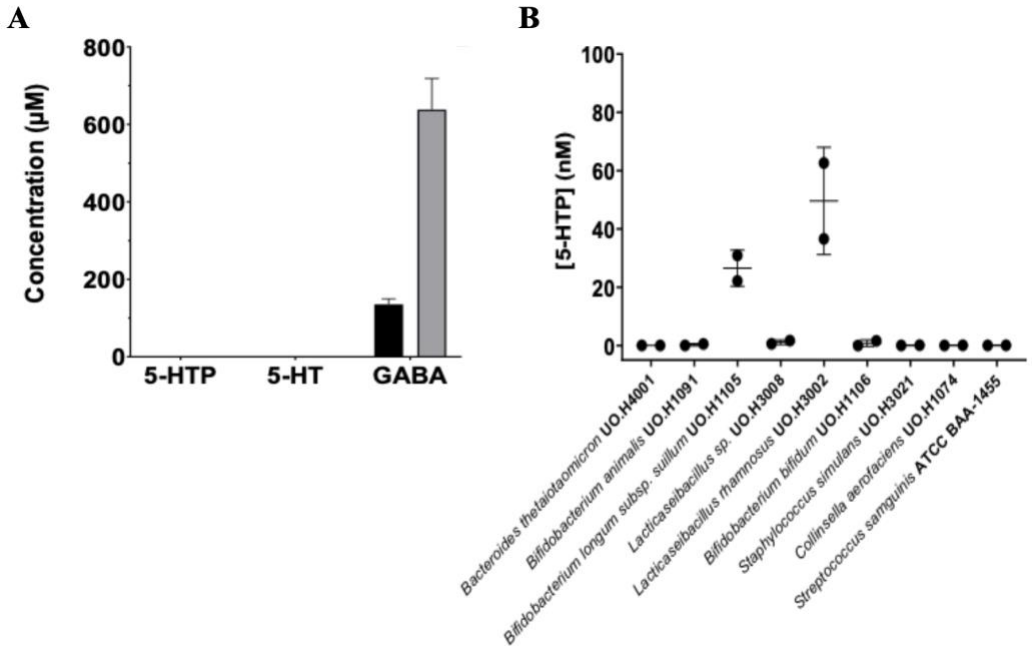

Figure 2.4. Metabolites quantification of selected strains with high enzyme activity. (A) Quantification of neuroactive metabolites produced by *Lacticaseibacillus rhamnosus* UO.H3002 (black) and *Bifidobacterium longum* subsp. *suillum* UO.H1105 (gray) using LC-MS/MS (µM). (B) Quantification of 5-HTP produced by selected strains using ELISA (nM).

3.3 Probiotic properties of L. rhamnosus UO.H3002 and B. longum subsp. suillum UO.H1105

3.3.2 Antibiotic susceptibility

The sensitivity of two selected strains, *L. rhamnosus* UO.H3002 and *B. longum* subsp. *suillum* UO.H1105 to different types of antibiotics is presented in **Table 2.1.** *L. rhamnosus* UO.H3002 showed a minimum inhibitory concentration (MIC) of <0.5 µg/mL for ampicillin, vancomycin, erythromycin, and tetracycline, indicating susceptibility (S) to these antibiotics. *L. rhamnosus* UO.H3002 showed MIC of 2 µg/mL for chloramphenicol which is below the breakpoint of 4 µg/mL, indicating susceptibility. As shown in **Table 2.1.** *L. rhamnosus* UO.H3002 was susceptible to most tested antibiotics except streptomycin and gentamycin, which showed intermediate

susceptibility of MIC 64 µg/mL and 16 µg/mL, respectively. *B. longum* subsp. *suillum* UO.H1105 exhibited resistance to several antibiotics, including chloramphenicol, vancomycin, gentamycin, streptomycin, and erythromycin, but was susceptible to ampicillin and tetracycline.

Table 2.1. Determination of MIC (Minimum Inhibitory Concentration) and sensitivity of potential probiotic candidates *Lacticaseibacillus rhamnosus* UO.H3002 and *Bifidobacterium longum* subsp. *suillum* UO.H1105 to antibiotics.

Antibiotics	***Lacticaseibacillus rhamnosus* UO.H3002**			***Bifidobacterium longum subsp. suillum* UO.H1105**		
	Breakpoints	**MIC (µg/mL)**	**Susceptibility**	**Breakpoints**	**MIC (µg/mL)**	**Susceptibility**
Chloramphenicol	4	2	S	4	32	R
Ampicillin	4	<0.5	S	2	<0.5	S
Vancomycin	n.r	<0.5	S	2	4	R
Gentamycin	16	16	S	64	512	R
Streptomycin	64	64	S	128	512	R
Erythromycin	1	<0.5	S	1	8	R
Tetracycline	2	<0.25	S	8	<2	S

3.3.1 Auto-aggregation assay

The auto-aggregation capacity of both the selected strains *L. rhamnosus* UO.H3002 and *B. longum* subsp. *suillum* UO.H1105 were measured at 2, 4, 16, and 24 h. The results showed distinct patterns with increasing auto-aggregation overtime as shown in **Figure 2.5**. For the *L. rhamnosus* UO.H3002, auto-aggregation began at (21.38% ± 3.49) at 2 h and slightly decreased to (20.47% ± 5.29) by 4 h. A substantial increase was observed at 16 h, with the auto-aggregation rising to (55.12% ± 3.88), followed by a further increase to (58.07% ± 0.44) at 24 h, indicating persistent aggregation over time. Conversely, *B. longum* subsp. *suillum* UO.H1105 exhibited a more fluctuating pattern. At 2 h, the auto-aggregation was similar to *L. rhamnosus* UO.H3002, (21.80% ± 5.08). However, it decreased significantly to (11.70% ± 5.04) at 4 h. By 16 h, the auto-

aggregation increased to (23.25% ± 5.31) and continued rising modestly to (25.52% ± 4.39) at 24 h. Overall, *L. rhamnosus* UO.H3002 showed substantial increase in aggregation compared to both positive control and *B. longum* subsp. *suillum* UO.H1105 as *B. longum* subsp. *suillum* UO.H1105 remained lower, and more variable.

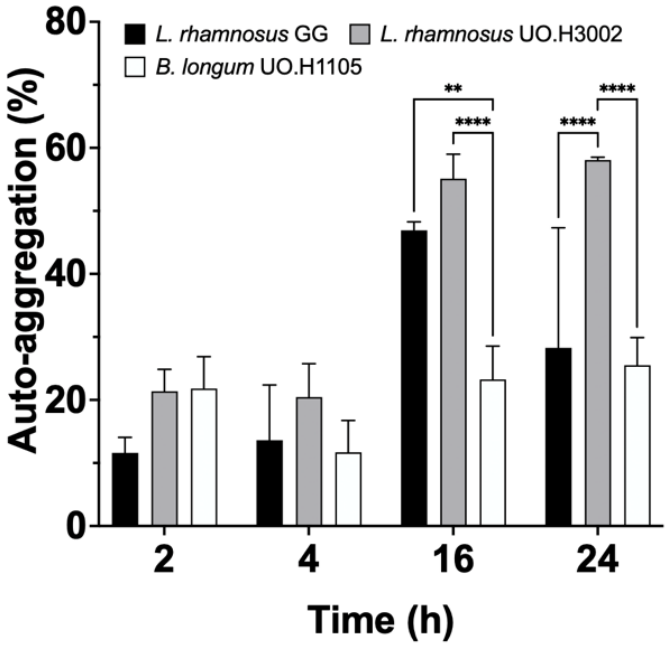

Figure 2.5. Auto-Aggregation percentages of potential probiotic candidates *Lacticaseibacillus rhamnosus* UO.H3002 (black) and *Bifidobacterium longum* subsp. *suillum* UO.H1105 (gray) at 2, 4, 16, and 24 hours of incubation at 37 °C. *Lacticaseibacillus rhamnosus* GG used as a reference probiotic strain (white). **p < 0.01, ****p < 0.0001.

3.3.3 Gastrointestinal and bile salt tolerance

Selected potential probiotic candidates were exposed to stimulated gastric and intestinal fluids to measure bacterial viability in the gastrointestinal tract. The initial concentration of the *L. rhamnosus* UO.H3002 was 8.70 ± 0.03 log CFU/mL, as shown in **Figure 2.6**. After exposure to pH 2.5 and pH 3.1, reduction in viability occurred, with the cell count decreasing to 4.95 ± 0.50 log CFU/mL and 8.76 ± 0.00 log CFU/mL, respectively. A further decline was observed following exposure to 0.3% bile salts, reducing the viable count to 4.78 ± 0.55 log CFU/mL, with survival rate more than 50% after three hours of exposure. In contrast, the *B. longum* subsp. s*uillum*

UO.H1105 began with a concentration of 8.60 log CFU/mL, exposure to pH 2.5 and pH 3.1 resulted maintain strong cell viability 8.55 ± 0.09 log CFU/mL and 8.7 ± 0.00 log CFU/mL respectively. However, similar to *L. rhamnosus* UO.H3002, exposure to 0.3% bile salts caused a reduction in the viable cell count to 6.6 ± 0.3 log CFU/mL, with survival rate more than 60% after 3 h of exposure. Despite differing responses to acidic conditions, both strains showed a notable tolerance for intestinal fluids.

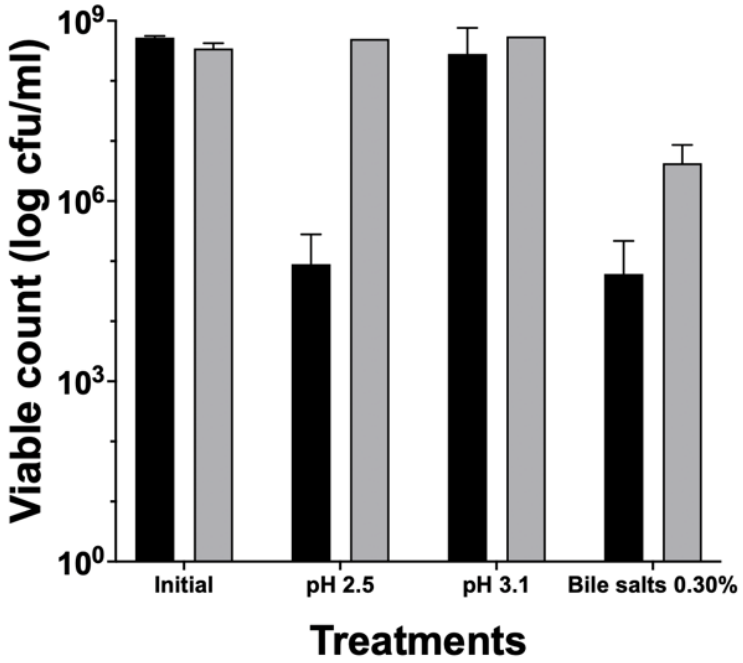

Figure 2.6. Viability of potential probiotic candidates *Lacticaseibacillus rhamnosus* UO.H3002 (black) and *Bifidobacterium longum* subsp. *suillum* UO.H1105 (gray) after exposure to simulated gastric and intestinal conditions.

3.4. Induction of 5-HT synthesis by enterochromaffin cells in vitro

We investigated the effects of selected probiotic candidates having high TPH enzyme activity and 5-HTP secretion on modulating 5-HT synthesis by enterochromaffin cells. RIN-14B cells were incubated for an hour with CFS from *L. rhamnosus* UO.H3002 or *B. longum* subsp. *suillum* UO.H1105 grown to late exponential phase. As shown in **Figure 2.7**, both tested strains did not upregulate *Tph1* gene, suggesting no induction of 5-HT secretion from enterochromaffin cells.

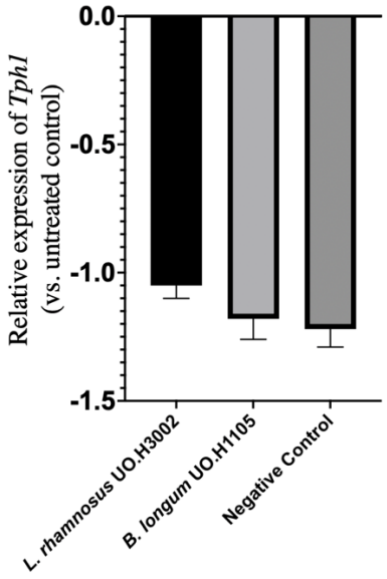

Figure 2.7. Gene expression of *Tph1* gene (key gene involved in serotonin biosynthesis) by RT-qPCR analysis after treatment of RIN14B cells with CFS recovered from *Lacticaseibacillus rhamnosus* UO.H3002 and *Bifidobacterium longum* subsp. *suillum* UO.H1105. Fold upregulation is shown after subtracting the expression from negative control (MRS supplemented with 1mM tryptophan).

4. Discussion

This study highlights the variability in TPH activity responsible for the hydroxylation of -L-tryptophan amino acids among gut microbial taxa and identify specific bacterial strains capable of 5-HTP synthesis, positioning them as promising probiotic candidates. Our findings emphasize the role of gut commensal microbes in regulating serotonin precursors, and suggest an alternative microbial source for 5-HTP production to meet growing demand [43,368,393].

The gut microbiota is increasingly recognized as key contributors to serotonin biosynthesis through direct and indirect mechanisms [8,32,256]. Gut microbiota mainly consist of six major phyla, *Bacillota* (formerly *Firmicutes*), *Bacteroidota* (formerly *Bacteroidetes*), *Actinomycetota* (formerly *Actinobacteria*), *Proteobacteria*, *Verrucomicrobia*, and *Fusobacteria*, with *Bacillota* and *Bacteroidota* accounting for 90% of the gut microbiota [227]. Consistent with previous studies, we observed that *Bacillota* phyla had the highest TPH activity. Several studies have found

that one of the hallmarks of depressive disorders is disturbance and reduction of *Bacillota* phylum [394–397]. Indeed, Li et al observed a positive correlation of *Bacillota* abundance and colonic 5-HT levels [398]. Insomnia and sleep disorders are also linked to dysfunction in serotonin levels, aging-adults with poor sleep were found to have reduced *Bacillota* abundance and 5-HTP supplements alleviated associated symptoms [399–404]. This suggests that the presence and balance of *Bacillota* in the gut play a critical role in maintaining serotonin synthesis, potentially influencing overall serotonergic function.

The phylum *Bacillota* comprises Gram-positive, spore-forming bacteria that are obligate anaerobes, exhibiting either a cocci or rod-shaped morphology. This group includes members of the *Enterococcaceae* and *Lactobacillaceae* families, as well as the *Streptococcus* genus [405]. We identified seven genera with markedly highest TPH enzyme activity, including *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, *Staphylococcus*, *Streptococcus*, and *Acidaminococcus*, and *Collinsella*, indicating that 5-HTP synthesis within the gut microbiota is not uniformly driven, rather heavily influenced by certain key players within the microbial community. Elevated TPH activity in lactic acid bacteria, such as *Bifidobacterium*, *Lactobacillus*, and *Enterococcus*, aligns with their established roles in serotonin regulation, with favorable impacts on various psychiatric disorders, including anxiety, depression, and stress-related behaviors [350,406–410]. The *Enterococcus* genus, in particular, has been shown to produce aromatic amines through aromatic amino acid decarboxylase, and stimulate colonic serotonin synthesis [150]. Specific species, such as *E. hirae*, *Enterococcus spp.*, and *E. gallinarum*, were found to directly synthesize 5-HT [411,412]. Whereas *E. faecalis* and *E. faecium* modulate serotonin levels via serotonin transporters [413]. Aligning with previous findings, we observed that *Enterococcus* genera is one of the most enzyme producers, particularly, *E. hirae*, *Enterococcus* spp., and *E. faecalis* exhibited the highest

TPH activity. Furthermore, in the current study, we observed that *Lactobacillus* and *Bifidobacterium* strains were among the highest strains that secreted 5-HTP in the supernatant, highlighting their significant role in serotonin biosynthesis. The high TPH activity observed in these strains supports their well-established involvement in gut-brain communication [256]. Notably, common *Lactobacillus* and *Bifidobacterium* strains found in the gut microbiota of both rats and humans have been shown to produce substantial levels of 5-HTP and serotonin (5-HT) in culture medium [9]. Additionally, previous research has detected the synthesis of serotonin and its metabolite, 5-hydroxyindoleacetic acid, in lactobacilli [148]. Consistent with these findings, Aizawa et al. (2016) reported a reduction in beneficial gut bacteria, including *Bifidobacterium* and *Lactobacillus*, in patients with major depressive disorder, further emphasizing the potential link between gut microbiota composition and serotonergic regulation [414]. Additionally, prior research has highlighted the roles of *Streptococcus* and *Staphylococcus* in serotonin-related pathways, strains belonging to both genera produce 5-HT, and while *Staphylococcus* also harbour staphylococcal aromatic amino acid decarboxylase utilized in 5-HTP metabolism to 5-HT [9,147,152,412]. Conversely, the associations involving *Acidaminococcus* and *Collinsella* with 5-HT biosynthetic pathways remain less explored.

To evaluate safety and health implications of strains with high 5-HTP producing bacteria, the probiotic potential of *L. rhamnosus* UO.H3002 and *B. longum subsp. suillum* UO.H1105, was confirmed. These strains demonstrated resilience to gastric and bile conditions, robust auto-aggregation, and sensitivity to antibiotics. The ability of probiotic strains to withstand harsh environments of the host is crucial to ensure it remains effective and can exert its beneficial effects without degradation. Generally, lactic acid strains categorized as Gram-positive bacteria widespread in nature including the human GI tract and found in various fermentable foods as well

as medicinal products that have the capacity to tolerate bile acid salt in the duodenum and stomach acid condition [415–417]. Studies have found that some *Lactobacillus* and *Bifidobacterium* strains possess proteins associated with bile acid resistance [418]. Multiple mechanisms contribute to the regulation of acid resistance, including neutralization processes, proton pumps, central metabolic pathways, and cellular alterations [419]. Bacterial auto-aggregation initiate adhesion process on intestinal mucosa, where the bacteria form a barrier to protect from external stressors and exert beneficial effects for the host [417,420]. It has been previously reported that *L. rhamnosus* and *B. longum* exhibited strong auto-aggregation properties [421,422]. In this study, we observed a survival rate of approximately 50% for *L. rhamnosus* UO.H3002 and over 60% for *B. longum subsp. suillum* UO.H1105 under simulated GI tract conditions. Additionally, similar to previous studies *L. rhamnosus* UO.H3002 exhibited strong auto-aggregation, with a rate of around 58% after 24 hours. Whereas *B. longum subsp. suillum* UO.H1105 showed a 25.52% aggregation rate. Furthermore, the susceptibility of probiotic strains to antibiotics is an essential factor in maintaining regulatory safety standards, to prevent the spread of antibiotic resistance genes and ensures their safe application in health-related products [423]. Generally, *Lactobacillus* species are naturally resistant to aminoglycosides (including streptomycin, kanamycin, gentamicin, and neomycin), ciprofloxacin, and trimethoprim, while remaining susceptible to penicillin, β-lactams, chloramphenicol, tetracycline, erythromycin, linezolid, and quinupristin-dalfopristin. While strains isolated from fermented foods have been found to attain resistance to tetracycline, erythromycin, clindamycin, and chloramphenicol [424]. In This study, *L. rhamnosus* UO.H3002, isolated from a newborn, was susceptible to all seven antibiotics tested (chloramphenicol, ampicillin, vancomycin, gentamicin, streptomycin, erythromycin, and tetracycline). In contrast, *B. longum subsp. suillum* UO.H1105, isolated from an adult donor, was only susceptible to ampicillin

and tetracycline. Previous research has shown that *Bifidobacterium* strains exhibit strain-dependent susceptibility to antibiotics, including ampicillin, tetracycline, chloramphenicol, and erythromycin [425]. Moubareck et al. further investigated antibiotic resistance in *Bifidobacterium*, screening 50 strains across eight species from human, animal, and probiotic sources. Their findings revealed minimal natural and acquired resistance to 30 antibiotics, with 14% of strains displaying tetracycline resistance [426]. The tet(W) gene, responsible for tetracycline resistance, was identified in *B. pseudocatenulatum* and *B. bifidum* and was also detected in *B. longum* and rumen obligate anaerobes, suggesting intergeneric transfer of resistance genes among anaerobic bacteria [426,427]. Duranti et al. concluded that antibiotic resistance in *Bifidobacterium* species is primarily acquired through horizontal gene transfer rather than vertical evolutionary inheritance [428].

There have been numerous reports on the intestinal microbial stimulation of 5-HT synthesis in enterochromaffin cells through various pathways including short-chain fatty acids, tryptophan indole metabolites, and secondary bile acids [8]. To evaluate the effects of microbial 5-HTP production on host 5-HT synthesis, selected strains, *L. rhamnosus* UO.H3002 and *B. longum subsp. suillum* UO.H1105*,* were incubated with enterochromaffin cell model, RIN14B cells. Although the duration was brief, the cells were pre-treated with fluoxetine, a selective serotonin reuptake inhibitor, to maximize the 5-HT concentration; this approach was adopted based on prior established methodology [294,333] demonstrated that *in-vivo* screening of probiotic strains involving supernatant treatment led to measurable *Tph1* gene expression. However, in the current study, neither *L. rhamnosus* UO.H3002 nor *B. longum* subsp. *suillum* UO.H1105 induced *Tph1* gene expression in enterochromaffin cells. This finding suggest that microbial 5-HTP secretion may not directly stimulate 5-HT synthesis in intestinal cells. Instead, microbial metabolites or

indirect pathways may mediate serotonin regulation, particularly through interactions with the central nervous system. Several studies denoted the detection of exogenous 5-HTP in brain regions, unlike 5-HT, is capable of crossing the blood-brain barrier and approximately 10% of the metabolites found in mammalian blood have gut microbial origin [5,429–434]. Alternative route to the brain may involve microbial extracellular vesicles (EVs), which are membrane-bound nano-sized particles being recognized as key mechanisms for microbiome-host communication [435]. Gut microbiome EVs could reach the brain, delivering several neurotransmitters and intermediate metabolites including GABA, glutamate, arachidonyl-dopamine, gabapentin, and N-acylethanolamines [365]. In addition, Zhang et al. [9] reported that increased 5-HTP production by gut microbiota led to elevated 5-HTP levels in the bloodstream, thereby increasing brain 5-HTP and 5-HT levels. Likewise, an oral dose of 5-HTP supplement elevated both 5-HTP and 5-HT levels in serotonergic neurons [436].

Despite these promising findings, several limitations must be acknowledged. While this study identified a clear correlation between bacterial taxa and TPH1 activity *in*-vitro, the direct effects of these microbial populations on *in*-vivo serotonin levels remain unconfirmed. The complex interplay among host factors, dietary patterns, and microbial communities requires further investigation to fully elucidate how these factors influence bacterial 5-HTP biosynthesis and the reciprocal effects of 5-HTP synthesis on microbiota composition. For instance, dietary tryptophan deficiency has been associated with microbiota dysbiosis and a decline in beneficial bacterial populations [437,438], whereas oral administration of 5-HTP has been shown to restore microbial dysbiosis, enhancing richness and diversity [403,439]. A recent study found that the production of specific tryptophan-derived metabolites is primarily influenced by the regulation of metabolic pathways in response to substrate availability (e.g., dietary fibers or nutrients) rather than by the

mere abundance of tryptophan-metabolizing bacterial populations, with fermentable fibers suppressing harmful indole production while promoting beneficial metabolites [289]. Moreover, exploring the impact of microbial 5-HTP on brain regions in inducing serotonin synthesis is crucial for a comprehensive understanding of this relationship.

Overall, this study underscores the variability in serotonin precursor synthesis and suggests that enhancing intestinal commensal bacteria through probiotic supplementation could potentially enhance intestinal health and support serotonin biosynthesis in the central nervous system, thereby positively influencing mood regulation and other serotonin-related functions, an area that merits further exploration.

5. Conclusion

In conclusion, this study adds to the growing body of literature on microbial serotonin synthesis by demonstrating that certain gut microbial residents play a more prominent role in serotonin biosynthesis, particularly through the production of the intermediate precursor 5-HTP. This insight holds promise for therapeutic strategies aimed at modulating serotonin pathway through targeted microbial interventions. Further research into microbiota-driven modulation of host serotonin synthesis could pave the way for probiotic-based treatments, with potential benefits for both gastrointestinal health and mental well-being.

Data availability

The 16S rRNA gene sequence of the bacterial isolates are available from the NCBI database (OP690542-OP690599).

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Author contributions

S.M. and R.H. conceived of and designed the study. S.A. and B.Y. isolated the gut bacteria and performed molecular identification. S.A. and S.M. prepared and processed the samples for enzyme activity and 5-HTP quantification. S.A. conducted assessment of probiotic properties. S.A. and N.N. conducted cell culture experiments. S.A. and Z.M. prepared and processed the samples for the metabolomic study. B.Y., and Z.M. analyzed the metabolomic data. S.A., S.M., G.A.E. analyzed and interpreted the data. S.A., S.M., G.A.E., and R.H. wrote or revised the manuscript. All authors reviewed and approved the final manuscript.

Declaration of competing interest

B.Y, S.M., and R.H. are co-inventors and co-applicants with the University of Ottawa on a patent application (US Patent Application Number 63/733,497, filed December 13, 2024) entitled 'GUT MICROBIOME PSYCHOBIOTICS, EXTRACELLULAR VESICLES THEREFROM, COMPOSITIONS COMPRISING THE SAME AND METHODS OF USING SAME'. The remaining authors declare no conflicts of interest. The funders had no role in the study design, collection, analyses, interpretation of data, writing of the manuscript, or decision to publish the results.

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Supplementary Materials

Table 2.S1. Bio-origin of intestinal isolates with respective culture media and conditions

Species/Identification	Growth Medium	Growth Condition
Bifidobacterium animalis UO.H1091	deMan, Rogosa, and Sharpe (MRS)+0.05%L-cystein	Anaerobic
Bifidobacterium bifidum UO.H1106	MRS+0.05%L-cystein	Anaerobic
Bifidobacterium animalis subsp. Lactis UO.H1107	MRS+0.05%L-cystein	Anaerobic
Bifidobacterium bifidum UO.H4023	MRS+0.05%L-cystein	Anaerobic
Bifidobacterium longum subsp. Suillum UO.H1105	MRS+0.05%L-cystein	Anaerobic
Bifidobacterium animalis subsp. UO.H1109	MRS+0.05%L-cystein	Anaerobic
Collinsella aerofaciens UO.H1074	Modified peptone-yeast extract glucose (MPYG)	Anaerobic
Collinsella aerofaciens UO.H1076	MPYG	Anaerobic
Collinsella aeofaciens UO.H1201	MPYG	Anaerobic
Staphylococcus simulans UO.H3021	MRS+0.05%L-cystein	Aerobic
Staphylococcus sp. UO.H3023	MRS+0.05%L-cystein	Aerobic
Staphylococcus epidermidis UO.H3020	MRS+0.05%L-cystein	Aerobic
Staphylococcus sp. UO.H3024	MRS+0.05%L-cystein	Aerobic
Enterococcus faecalis UO.H3015	Brain Heart Infusion (BHI)+0.05%L-cystein	Aerobic
Enterococcus hirae UO.H3016	BHI+0.05%L-cystein	Aerobic
Enterococcus sp. UO.H3018	BHI+0.05%L-cystein	Aerobic
Enterococcus faecalis UO.H3009	BHI+0.05%L-cystein	Aerobic
Enterococcus faecalis UO.H3010	BHI+0.05%L-cystein	Aerobic
Enterococcus hirae UO.H3017	BHI+0.05%L-cystein	Aerobic
Enterococcus faecalis UO.H3011	BHI+0.05%L-cystein	Aerobic
Enterococcus faecalis UO.H3014	BHI+0.05%L-cystein	Aerobic
Enterococcus sp. UO.H3019	BHI+0.05%L-cystein	Aerobic
Enterococcus faecalis UO.H3012	BHI+0.05%L-cystein	Aerobic
Enterococcus faecalis UO.H3013	BHI+0.05%L-cystein	Aerobic
Eubacterium rectale ATCC 33656	BHI	Anaerobic
Acidaminococcus intestine UO.H1097	MPYG	Anaerobic
Paenibacillus glucanolyticus UO.H1100	BHI	Anaerobic
Clostridium perfringens UO.H4031	Fastidious Anaerobe Broth (FAB)	Anaerobic
Clostridium leptum ATCC 29065	FAB	Anaerobic
Faecalibacterium prausnitzii ATCC 27768	FAB	Anaerobic
Lactobacillus casei ATCC 393	MRS+0.05%L-cystein	Aerobic
Lactobacillus rhamnosus ATCC 53103	MRS+0.05%L-cystein	Aerobic
Lactobacillus rhamnosus UO.H3002	MRS+0.05%L-cystein	Aerobic
Lactobacillus sp. UO.H3008	MRS+0.05%L-cystein	Aerobic
Lacticaseibacillus rhamnosus UO.H1094	MRS+0.05%L-cystein	Aerobic
Lactobacillus rhamnosus UO.H3007	MRS+0.05%L-cystein	Aerobic
Lactobacillus reuteri ATCC 23272	MRS+0.05%L-cystein	Aerobic
Lactobacillus rhamnosus UO.H3006	MRS+0.05%L-cystein	Aerobic
Lactobacillus rhamnosus UO.H3004	MRS+0.05%L-cystein	Aerobic
Lactobacillus rhamnosus UO.H3005	MRS+0.05%L-cystein	Aerobic
Lactobacillus rhamnosus UO.H3003	MRS+0.05%L-cystein	Aerobic
Lactobacillus rhamnosus UO.H3001	MRS+0.05%L-cystein	Aerobic
Streptococcus salivarius ATCC 27853	MRS+0.05%L-cystein	Aerobic

Streptococcus samguinis ATCC BAA-1455	MRS+0.05%L-cystein	Aerobic
Bacteroides sp. UO.H4010	FAB	Anaerobic
Bacteroides faecis UO.H4038	FAB	Anaerobic
Bacteroides sp. UO.H4014	FAB	Anaerobic
Bacteroides sp. UO.H4028	FAB	Anaerobic
Bacteroides sp. UO.H4034	FAB	Anaerobic
Bacteroides sp. UO.H4037	FAB	Anaerobic
Bacteroidaceae species UO.H1003	FAB	Anaerobic
Bacteroides dori UO.H4004	FAB	Anaerobic
Bacteroides finegoldii UO.H1052	FAB	Anaerobic
Bacteroides stercoris UO.H1036	FAB	Anaerobic
Bacteroides dorei UO.H1032	FAB	Anaerobic
Bacteroides thetaiotaomicron UO.H4001	FAB	Anaerobic
Bacteroides faecis UO.1051	FAB	Anaerobic
Bacteroides ovatus UO.H1053	FAB	Anaerobic
Bacteroides zhangwenhongii UO.H1055	FAB	Anaerobic
Bacteroides vulgatus UO.H1016	FAB	Anaerobic
Bacteroides caccae UO.H2003	FAB	Anaerobic
Bacteroides stercoris UO.H2001	FAB	Anaerobic
Bacteroides uniformis UO.H1043	FAB	Anaerobic
Bacteroides cellulosilyticus UO.H1027	FAB	Anaerobic
Bacteroides dori UO.H4005	FAB	Anaerobic
Phocaeicola vulgatus UO.H4018	FAB	Anaerobic
Phocaeicola sp. U.OH4019	FAB	Anaerobic
Phocaeicola dori UO.H4020	FAB	Anaerobic
Phocaeicola vulgatus UO.H4033	FAB	Anaerobic
Phocaeicola vulgatus UO.H4035	FAB	Anaerobic
Phocaeicola vulgatus UO.H4036	FAB	Anaerobic
Phocaeicola vulgatus UO.H4013	FAB	Anaerobic
Phocaeicola vulgatus UO.H4003	FAB	Anaerobic
Phocaeicola vulgatus UO.H1024	FAB	Anaerobic
Phocaeicola massiliensis UO.1001	FAB	Anaerobic
Parabacteroides johnsonii UO.H1049	FAB	Anaerobic
Alistipes putredinis UO.H1057	FAB	Anaerobic
Butyricimonas virosa UO.H1056	FAB	Anaerobic
Pseudomonas aeruginosa ATCC 27853	BHI	Anaerobic
Escherichia coli ATCC 25922	BHI	Anaerobic
Fusobacterium nucleatum ATCC	BHI	Anaerobic
Akkermansia muciniphila ATCC BAA-835	BHI+0.5% Mucin	Anaerobic

CHAPTER 3:

General Discussion and Conclusion

This thesis project explored the intricate relationship between gut microbiota and serotonin biosynthesis, emphasizing microbial contributions to serotonin precursor availability in gut environment. In this study, we screened various intestinal microbial taxa representative of healthy gut microbiota for TPH activity, an enzymatic reaction responsible for the hydroxylation of L-tryptophan to 5-HTP. The findings highlight the potential role of specific bacterial taxa in regulating serotonin precursors, highlighting a novel avenue for microbiota-based therapeutic strategies.

The main findings emphasize the significant variability in microbial taxa with only a subset of bacterial taxa demonstrating significant TPH activity. We identified differences in TPH activity among three primary phyla in human intestine including *Bacillota*, *Actinobacteria,* and *Bacteroidota* with *Bacillota* significantly showcasing the highest activity. Two bacterial strains *Lacticaseibacillus rhamnosus* UO.H3002 and *Bifidobacterium longum* subsp. *suillum* UO.H1105 were identified with notable 5-HTP biosynthesis capabilities and interesting probiotic features, indicating their strong potential as psychobiotic candidates. In This study, *L. rhamnosus* UO.H3002, isolated from a newborn, was susceptible to all tested antibiotics, demonstrated resilience to gastric and bile conditions, robust auto-aggregation, and 5-HTP production. These findings align with growing evidence that gut commensal microbes can exert systemic effects on both gastrointestinal and central nervous system functions, influencing GI tract motility, mood, and cognition [440].

While we identified high 5-HTP producers, the study also raises important questions regarding the mechanisms by which bacterial 5-HTP production translates into host serotonergic regulation. Since microbial-derived 5-HTP represents a direct precursor for serotonin synthesis, the extent to which it influences host serotonin pools remains unclear. Additionally, variability in

TPH activity could be attributed to various factors. Albeit all bacteria media were supplemented with the same concentration of tryptophan. An often-overlooked factor in microbial modulation of serotonin synthesis is bacterial *de novo* tryptophan synthesis, rather than relying solely on supplements/dietary sources. Certain bacterial species possess tryptophan biosynthetic pathways, and the presence or absence of these pathways may play a core factor in bacterial TPH activity [441–443]. As strains with a greater capacity for tryptophan synthesis may exhibit more consistent or enhanced TPH activity and 5-HTP secretion. For instance, genomic analysis revealed that potential probiotic candidate *Lacticaseibacillus rhamnosus* UO.H3002 was found to possess multiple genes involved in tryptophan synthesis. Furthermore, it is equally critical to consider alternative pathways, such as plant-like mechanisms, in understanding the broader landscape of microbial 5-HTP production. It is plausible that bacteria exhibiting the highest TPH activity predominantly secrete 5-HTP via TPH modulation, while strains with lower TPH activity may rely on plant-like pathways for 5-HT synthesis that involves the decarboxylation of tryptophan to tryptamine followed by hydroxylation.

The complexity of the intestinal microenvironment is critical in shaping serotonin synthesis and utilization. Tryptophan being an essential precursor for serotonin synthesis, its metabolic fate is influenced by microbial activity. Some bacterial species divert tryptophan toward the kynurenine and other metabolic pathways, thereby reducing its availability for serotonin production, which may subsequently affect gut motility and contribute to mood disorders. At the same time, serotonin itself modulates microbial composition by influencing gut motility, creating a selective environment for specific bacterial populations. This intricate feedback loop highlights a dynamic and adaptive system that responds to physiological needs and external influences, carrying significant clinical implications. Previous literature has established a strong link between microbial

dysbiosis and conditions such as irritable bowel syndrome (IBS), depression, and anxiety, underscoring the importance of 5-HT therapeutic strategies aimed at restoring microbial balance. Dietary interventions, prebiotics and probiotics have shown promising results (summarized in Table 1.1) in modulating gut microbiota composition and optimizing serotonin-mediated functions, potentially improving overall health. However, despite extensive research on the effects of serotonin on host physiology, relatively little is known about the direct impact of microbial-derived 5-HTP on intestinal health and broader host functions. Addressing these gaps are essential for advancing our understanding of microbial contributions to serotonin biosynthesis and their potential therapeutic applications. Furthermore, despite advancement in microbial isolation techniques certain bacterial species remain challenging to cultivate in laboratory conditions, limiting our ability to fully characterize their roles in serotonin metabolism. To overcome this limitation, *ex-vivo* and *in-vivo* models may provide valuable tools for examining bacterial 5-HTP synthesis under physiologically relevant conditions. These models not only offer insights into the intrinsic and environmental factors influencing microbial serotonergic pathways but also enhance the ability to screen gut microbiome on a larger scale with significant contributions to host serotonin regulation.

The multifaceted interactions between microbial metabolites, serotonin pathways, and host neurochemical balance, are fundamental to maintaining an optimal health. Microbial communities residing in the gut can directly synthesis and metabolize tryptophan into diverse array of metabolites that then directly or in-directly contribute to serotonin synthesis. Understanding the connection between microbial ecology and neurochemical processes may provide valuable insights into conditions where serotonin levels are disrupted and reinforce the importance of a healthy gut microbiota. Continued research in this area is crucial to fully elucidate the mechanisms

by which microbial communities influence serotonin biosynthesis and to explore the therapeutic potential of microbiome-based interventions. Future key areas of investigation include:

1- Examining the effects of microbial communities and host factors on tryptophan hydroxylase activity through GI tract simulation and animal models.
2- Exploring the delivery mechanisms (e.g. extracellular vesicles) of bacterial 5-HTP to host including the gut-brain axis.
3- Conducting *in-vivo* studies assessing the validity and impact of bacterial 5-HTP secretion on the intestinal microenvironment and host.
4- Determining optimal dietary and environmental conditions that enhance microbial 5-HTP synthesis via precision nutrition approaches.
5- Translating clinical findings into human application through microbiome-based interventions for serotonin dysregulation by investigating whether 5-HTP producer probiotic strains can induce changes in gut-derived and systemic serotonin markers and can effectively modulate serotonergic system in individuals with depression, anxiety, IBS, or other serotonin-related disorders.

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